Ferredoxin-dependent electron transport during methanogenesis from acetate

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Cornelia Welte

aus

Frechen

Bonn, 2011

Angefertigt mit Genehmigung der Mathematisch Naturwissenschaftlichen Fakultät
der

Rheinischen Friedrich-Wilhelms-Universität Bonn

1. Gutachter: Prof. Dr. Uwe Deppenmeier

2. Gutachterin: apl. Prof. Dr. Christiane Dahl

Tag der Promotion: 06.07.2011

Erscheinungsjahr: 2011

Teile dieser Arbeit wurden bereits veröffentlicht.

Welte C, Kallnik V, Grapp M, Bender G, Ragsdale S, Deppenmeier U (2010) Function of Ech hydrogenase in ferredoxin-dependent, membrane-bound electron transport in Methanosarcina mazei. *J Bacteriol* **192**:674-678.

Welte C, Krätzer C, Deppenmeier U (2010) Involvement of Ech hydrogenase in energy conservation of *Methanosarcina maze*i. *FEBS J* **277**:3396-3403.

Welte C, Deppenmeier U (2011) Re-evaluation of the function of the F_{420} dehydrogenase in electron transport of *Methanosarcina mazei*. *FEBS J* **278**:1277-1287.

Welte C, Deppenmeier U (2011) Proton translocation in methanogens. *Methods Enzymol* **494**:257-280.

Welte C, Deppenmeier U (2011) Membrane-bound electron transport in *Methanosaeta thermophila*. *J Bacteriol* **193**:2868-2870.

Table of contents

Chapter 1

Introduction to methanogenic archaea and methanogenesis

Ecology, phylogeny and distribution of methanogens

Methanogenic archaea are one of the key groups in the global carbon cycle because they metabolize the final products of aerobic and anaerobic respirations and fermentations. The need of every ecosystem to maintain an intact carbon cycle makes methanogens widespread in nature with many habitats commonly known: the methanogens are found in anaerobic sediments of paddy fields, swamps and other wetlands. Methane is the final product of methanogenesis, and due to the occurrence of methanogens in wetlands, methane has also been described as "swamp gas". Methanogens are also present in sediments and other oceanic anaerobic environments. In the deep sea they are well known for being responsible for the formation of methane hydrates. Furthermore, the digestive tract of many animals is inhabited by methanogens, with ruminants as most prominent example, and every year cattle livestock emit considerable amounts of methane into the atmosphere. Although the atmospheric methane concentration is much lower than that of CO_2, methane contributes considerably to global warming because it is 21-fold more active as a greenhouse gas than CO_2. Extensive use of agriculture and cattle livestock has increased the atmospheric methane concentration by over 150 % in the last 250 years (WMO Greenhouse Gas Bulletin, 2009), so the understanding of methanogenesis is now more important than ever. Additionally, methanogens are biotechnologically employed in the production of biogas – methane – in wastewater treatment and biogas plants and are thus involved in the generation of renewable energy sources.

Six orders of methanogens are known: *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanocellales* and *Methanosarcinales*. The first five orders have a limited substrate spectrum. All but one known species can grow on H_2+CO_2 or formate, whereas members of the order *Methanosarcinales* have a broad substrate spectrum including acetate, methylated amines or sulfides, methanol and H_2+CO_2. The order *Methanosarcinales* is composed of two families (Kendall and Boone, 2006), the *Methanosaetaceae* and the *Methanosarcinaceae*. The family *Methanosaetaceae* comprises only one genus (*Methanosaeta*) that is restricted to growth on acetate. The family *Methanosarcinaceae* is composed of six genera, of which five are restricted to growth on methylated amines and methanol and are incapable of growth either on acetate or H_2+CO_2. Members of the sixth

genus – *Methanosarcina* – are capable of growth on acetate, methylated amines, methanol and sometimes H_2+CO_2 and can be regarded as the metabolically most versatile genus of all methanogens.

The methanogenic substrate acetate is metabolized in the so-called aceticlastic pathway. Aceticlastic methanogenesis is thought to be the major contributor to biogenic methane emission with up to 70 % of the global biogenic methane originating from aceticlastic methanogens (Ferry and Lessner, 2008). There are only two genera that are able to grow aceticlastically, both belong to the *Methanosarcinales*: *Methanosarcina* sp. are facultatively aceticlastic and *Methanosaeta* sp. are obligately aceticlastic. Regarding the occurrence of both genera in natural habitats, they differ in their minimal threshold concentration of acetate: *Methanosarcina* sp. grow in environments containing at least 0.2 – 1.2 mM acetate whereas *Methanosaeta* sp. can already grow with 7 – 70 µM (Jetten *et al.*, 1992b). The differences in the acetate threshold concentrations is also reflected in their occurrence in biogas facilities whose community composition has been intensively studied (Franke-Whittle *et al.*, 2009; Lee *et al.*, 2009a; Lee *et al.*, 2009b; Shin *et al.*, 2010; Supaphol *et al.*, 2011). Furthermore, *Methanosaeta* sp. were found to be responsible for reactor performance and stability of the biogas plants at low acetate concentrations (Karakashev *et al.*, 2005; Supaphol *et al.*, 2011). The physiological understanding of aceticlastic methanogenesis, and thus the physiology of *Methanosarcina* and *Methanosaeta* sp., is of special importance and will be the main subject of this work.

Methanogenesis – central to all methanogens

The central pathway of methanogenesis is well conserved among all methanogenic archaea, and has been thoroughly investigated in past decades. In principal, three pathways of methanogenesis can be described: methylotrophic, aceticlastic and hydrogenotrophic methanogenesis. Methylotrophic methanogens utilize methyl groups deriving from methylated amines, sulfides or methanol. Aceticlastic methanogens gain energy from the metabolization of acetate, and hydrogenotrophic methanogens use H_2+CO_2 for energy conservation.

In methylotrophic methanogenesis (Figure 1) methyl groups are transferred to coenzyme M (CoM, 2-mercaptoethanesulfonate) by substrate-specific methyl

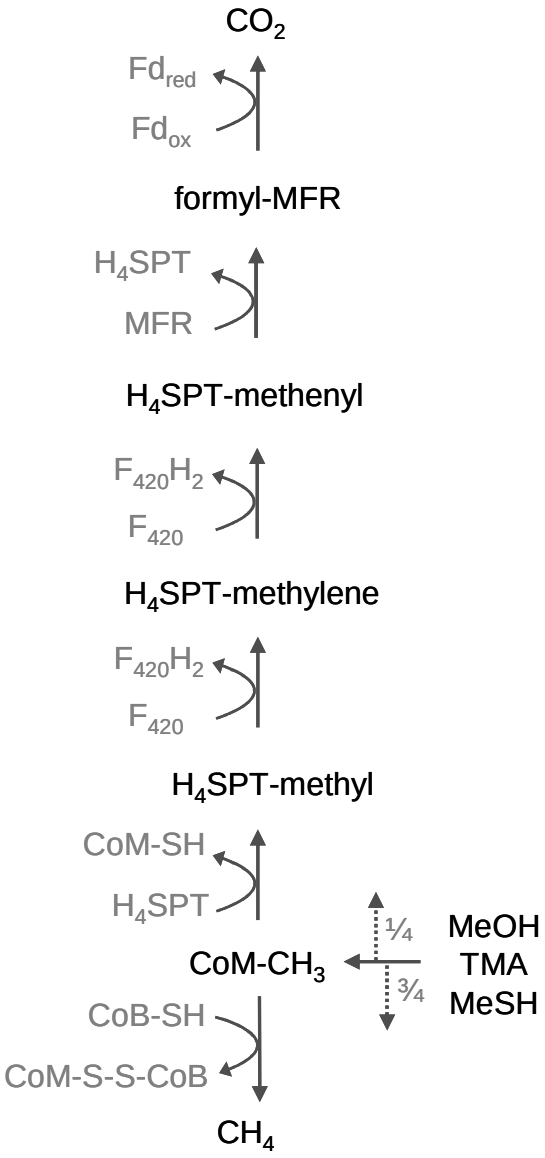

Figure 1: Methylotrophic methanogenic pathway. Methyl groups enter the central methanogenic pathway at the stage of CoM and are then reduced to CH_4 or oxidized to CO_2. In the course of these reactions, reducing equivalents ($Fd_{red}/F_{420}H_2$) and the terminal electron acceptor heterodisulfide (CoM-S-S-CoB) are built. Fd_{red}/Fd_{ox}, reduced/oxidized ferredoxin; H_4SPT, tetrahydrosarcinapterin; MeOH, methanol; MFR, methanofuran; TMA, trimethylamine; MeSH, methylsulfide.

transferases (Ferguson *et al.*, 2000; Tallant *et al.*, 2001; Pritchett and Metcalf, 2005; Krätzer *et al.*, 2009). One out of four methyl groups is oxidized to CO_2 to yield reducing equivalents, and three out of four methyl groups are reduced to methane. The production of methane is accompanied by the oxidative coupling of CoM to coenzyme B (CoB, N-7-mercaptoheptanoyl-L-threonine phosphate). The resulting heterodisulfide (CoM-S-S-CoB) is the terminal electron acceptor of the methanogenic respiratory chain. In the oxidative branch of methanogenesis the

Methanosarcina mazei

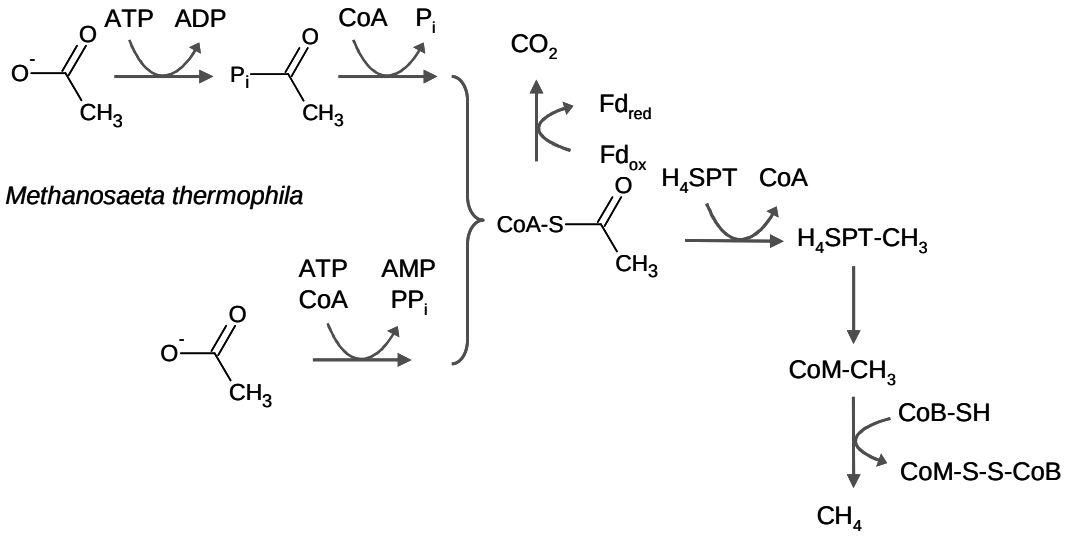

Figure 2: Aceticlastic methanogenic pathway. Acetate activation in *Ms. mazei* and *Mt. thermophila* is different (left) but the resulting acetyl-CoA is probably metabolized similarly (right). Fd_{red}/Fd_{ox}, reduced/oxidized ferredoxin; H_4SPT, tetrahydrosarcinapterin.

methyl group of CoM is transferred to tetrahydrosarcinapterin (H_4SPT) by the action of a membrane-bound methyl transferase that translocates two Na^+ per methyl group into the cytoplasm (Becher *et al.*, 1992; Lienard *et al.*, 1996). The H_4SPT-bound methyl ($-CH_3$) group is oxidized to the level of methylene ($-CH_2$) and formyl (-CHO), and the electrons are transferred to the 8-hydroxy-5-deazaflavin cofactor F_{420} to give two molecules of the reduced cofactor, $F_{420}H_2$. The formyl-group is transferred to methanofuran (MFR), and the resulting formyl-MFR is a substrate for the formyl-MFR dehydrogenase. In this process, two electrons are transferred to ferredoxin, and CO_2 is released (Kaesler and Schönheit, 1989; de Poorter *et al.*, 2003).

In aceticlastic methanogenesis, acetate first has to be activated. The mode of activation differs among *Methanosarcina* and *Methanosaeta* sp. (Figure 2). In *Methanosarcina* sp., acetate is phosphorylated to acetyl phosphate, and then the phosphate group is substituted by coenzyme A (CoA) (Ferry, 1997). *Methanosaeta* sp. seem to employ acetyl-CoA synthetases that split ATP to AMP + PP_i (Jetten *et al.*, 1989; Allen and Zinder, 1996). In comparison, *Methanosarcina* sp. hydrolyze one ATP equivalent for acetate activation whereas the expense for acetate activation in *Methanosaeta* sp. is two ATP equivalents. *Methanosaeta* sp. lack a membrane-bound, ion-translocating pyrophosphatase (Smith and Ingram-Smith, 2007) and the analysis of the soluble pyrophosphatase indicates that the formed pyrophosphate is hydrolyzed without contributing to substrate-level or

electron-transport phosphorylation (Jetten *et al.*, 1992a, Stefanie Berger (University of Bonn), personal communcation).

The intermediate that is common for acetate activation of *Methanosarcina* and *Methanosaeta* sp. is acetyl-CoA (Figure 2). Acetyl-CoA is a substrate for a CO dehydrogenase/acetyl-CoA synthase that cleaves the acetyl-CoA molecule: the carbonyl group is oxidized to CO_2, and two electrons are transferred to ferredoxin. The methyl group enters the reductive branch of methanogenesis and is transferred to H_4SPT. The membrane-bound methyl transferase acts in the reverse direction as compared to methylotrophic methanogenesis and conserves energy in the form of two Na^+ per methyl group transferred to CoM. Finally CH_4 is released by the action of the methyl-CoM reductase, with concomitant heterodisulfide production. In total, two electrons are transferred to ferredoxin and one molecule of the heterodisulfide is formed by the catabolism of one acetate molecule. In this process, two Na^+ per acetate molecule contribute to the electro-chemical gradient, and one (*Methanosarcina* sp.) or two (*Methanosaeta* sp.) ATP equivalents are hydrolyzed in the activation reaction. It is important to note that in aceticlastic methanogenesis F_{420} is not reduced and all electrons are transferred to ferredoxin.

Hydrogenotrophic methanogenesis is not a topic of this work and will be discussed only very briefly. Hydrogenotrophic methanogens reduce CO_2 to CH_4 with electrons deriving from H_2. Therefore, they make use of different hydrogenases: the soluble F_{420} hydrogenase generates reduced F_{420} whereas the reduction of ferredoxin is performed by different enzymes in obligate and facultative hydrogenotrophic methanogens. Obligate hydrogenotrophs contain a soluble hydrogenase (Mvh) that forms a complex with a soluble heterodisulfide reductase (HdrABC). Only recently has it been shown that ferredoxin reduction by this complex is connected to heterodisulfide reduction by electron bifurcation of the electrons deriving from H_2 (Kaster *et al.*, 2011). The coupling of the exergonic reduction of the heterodisulfide to the endergonic reduction of ferredoxin drives this reaction. In the facultative hydrogenotrophic *Methanosarcina* sp. ferredoxin reduction is not performed by the Mvh/Hdr complex. These organisms make use of the membrane-bound Ech hydrogenase (Meuer *et al.*, 2002).

All methanogenic pathways lead to the formation of the terminal electron acceptor heterodisulfide (CoM-S-S-CoB) and reducing equivalents. Endogenously produced reducing equivalents are reduced ferredoxin, the reduced form of F_{420} ($F_{420}H_2$) or molecular hydrogen, depending on the methanogenic pathway used. Molecular hydrogen can be additionally supplied by exogenous sources. These reducing

equivalents together with the heterodisulfide can be used in the methanogenic respiratory chain to generate an electro-chemical ion gradient.

Respiration in *Methanosarcina* and *Methanosaeta* sp.

Energy conservation in methanogenic archaea is performed by electron-transport phosphorylation that can be defined as heterodisulfide respiration. In *Methanosarcina* sp. different heterodisulfide oxidoreductase systems exist that constitute a branched respiratory chain (Figure 3).

The terminal reductase – the heterodisulfide reductase – is common to all systems and is well studied (Heiden *et al.*, 1994; Künkel *et al.*, 1997; Simianu *et al.*, 1998; Ide *et al.*, 1999; Madadi-Kahkesh *et al.*, 2001; Murakami *et al.*, 2001; Hedderich *et al.*, 2005). It consists of a membrane-integral *b*-type cytochrome (HdrE) and a membrane-associated [4Fe4S] cluster containing protein (HdrD). The heme groups in HdrE accept electrons from the membrane-soluble electron carrier methanophenazine (Abken *et al.*, 1998) that are used by HdrD for the reduction of the heterodisulfide to the corresponding thiols, CoM-SH and CoB-SH. In this process, 2 H^+ / 2 e^- are translocated across the cytoplasmic membrane (Ide *et al.*, 1999).

The formation of a reduced methanophenazine pool is accomplished by two membrane-integral protein complexes, the $F_{420}H_2$ dehydrogenase (Ide *et al.*, 1999; Bäumer *et al.*, 2000) and the F_{420} non-reducing hydrogenase. The $F_{420}H_2$ dehydrogenase (Fpo, $\mathbf{F}_{420}$: **p**henazine **o**xidoreductase) is composed of 13 subunits (FpoABCDFHIJKLMNO) and shows high homologies to the bacterial **N**ADH: **u**biquinone **o**xidoreductase (Nuo), also known as complex I. The most striking differences are (i) the replacement of the bacterial electron input module NuoEFG with FpoF that enables the complex to use $F_{420}H_2$ instead of NADH and (ii) the alteration of the quinone binding pocket in FpoD so that methanophenazine instead of quinone is bound (Deppenmeier, 2002a). It was suggested that in *Methanosarcina* (*Ms.*) *barkeri*, in contrast to *Ms. mazei*, the cytoplasmic $\mathbf{F}_{420}$ **r**educing **h**ydrogenase Frh instead of Fpo is responsible for $F_{420}H_2$ oxidation (Kulkarni *et al.*, 2009). To clarify this issue the role of Frh and Fpo was investigated in this work together with possible additional function(s) of Fpo and parts thereof. The Fpo complex contains FAD and various [4Fe4S] clusters that allow the enzyme to transport electrons to the methanophenazine pool, with concomitant proton translocation (2 H^+ / 2 e^-).

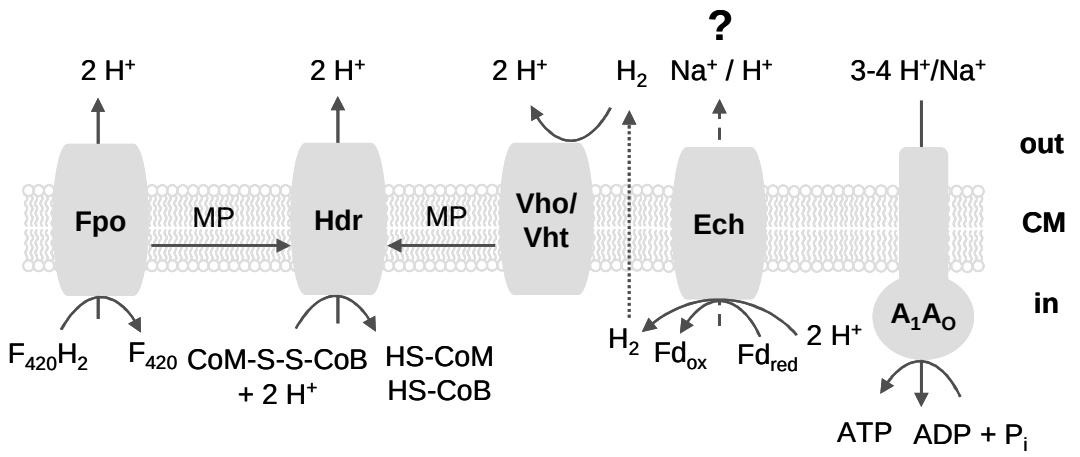

Figure 3: Model of the respiratory chain of *Ms. mazei* at the beginning of this work. Ion translocation by Ech hydrogenase is putative. A_1A_O, ATP synthase; Ech, Ech hydrogenase; Hdr, heterodisulfide reductase; Fd_{red}/Fd_{ox}, reduced/oxidized ferredoxin; Fpo, $F_{420}H_2$ dehydrogenase; MP, methanophenazine; Vho/t, F_{420} non-reducing hydrogenase (isoforms one and two).

The F_{420} non-reducing hydrogenase Vho/Vht (**v**iologen **h**ydrogenase **o**ne / **t**wo) does not interact with F_{420} but is used when H_2 serves as electron donor (Ide *et al.*, 1999). This enzyme consists of three subunits, two of which are located at the extracellular side of the cytoplasmic membrane and represent the typical large and small subunit of [NiFe] hydrogenases. They are anchored in the membrane by a *b*-type cytochrome that transfers electrons to methanophenazine. Two protons are released to the extracellular side when the H_2 molecule is oxidized, and methanophenazine takes up two protons upon reduction, so a vectorial proton translocation with the stoichiometry of 2 H^+ / 2 e^- can be observed (Ide *et al.*, 1999).

The ferredoxin oxidizing enzyme system differs among the *Methanosarcinales*: *Ms. mazei* and *Ms. barkeri* employ Ech hydrogenase (Meuer *et al.*, 1999; Welte *et al.*, 2010a; Welte *et al.*, 2010b) whereas it is hypothesized that the Rnf complex substitutes for Ech hydrogenase in *Ms. acetivorans* (Ferry and Lessner, 2008). In *Methanosaeta* (*Mt*.) *thermophila*, neither Ech hydrogenase nor the Rnf complex is encoded in the genome, and nothing is known about ferredoxin-dependent electron transport. Table 1 summarizes the presence of genes encoding hitherto mentioned membrane-bound protein complexes in members of the genera *Methanosarcina* and *Methanosaeta* with published genome sequences.

In *Ms. mazei* and *Ms. barkeri* Ech hydrogenase is responsible for membrane-bound ferredoxin oxidation. Ech hydrogenase is a [NiFe] hydrogenase that belongs to the subgroup of multisubunit [NiFe] hydrogenases. Multisubunit [NiFe] hydrogenases are thought to couple hydrogen formation in the course of ferredoxin oxidation with

Table 1: Occurrence of membrane-bound enzymes possibly involved in energy conservation in sequenced members of the genera *Methanosarcina* (*Ms.*) and *Methanosaeta* (*Mt.*).

Strain	Vho	Rnf	Ech	Fpo	HdrDE	Source
Ms. mazei	+	–	+	+	+	Deppenmeier *et al.*, 2002
Ms. barkeri	+	–	+	+	+	Maeder *et al.*, 2006
Ms. acetivorans	–[(1)]	+	–	+	+	Galagan *et al.*, 2002
Mt. thermophila	–	–	–	+[(2)]	+	Smith and Ingram-Smith, 2007

[(1)] Genes coding for *vho* present, but not expressed (Guss *et al.*, 2009; Rohlin and Gunsalus, 2010).

[(2)] Fpo complex is incomplete, F-subunit is missing (Smith and Ingram-Smith, 2007).

ion translocation. The initial designation “Ech” by Meuer et al 1999 was inspired by its homology to the ***E****scherichia* ***c****oli* **h**ydrogenase 3. Later the abbreviation “Ech” was sometimes described as **e**nergy-**c**onserving **h**ydrogenase (Hedderich and Forzi, 2005), although an energy-conserving function had not yet been experimentally demonstrated. The characterization of a *Ms. mazei* Δ*ech* mutant and the biochemical investigation of the ion-translocating function of Ech hydrogenase will be a central topic of this work.

In contrast, energy conservation in *Methanosaeta* sp. has hardly been investigated to date. The analysis of the genome sequence of *Mt. thermophila* (Smith and Ingram-Smith, 2007) did not elucidate electron transport mechanisms because known membrane-bound electron input proteins are missing: neither Ech hydrogenase nor the Rnf complex is encoded in the genome. So if ferredoxin serves as electron donor to the *Mt. thermophila* respiratory chain a novel type of oxidoreductase has to be involved. The investigation of electron transport processes in *Mt. thermophila* will also be examined in this work.

Finally, the electro-chemical gradient built up by the systems described above can be used for energy conservation. Energy is conserved by the action of an A_1A_O ATP synthase. In *Ms. mazei*, the ATP synthase is driven either by the proton motive force (Pisa *et al.*, 2007) or by the sodium motive force (Katharina Schlegel (University of Frankfurt), personal communication). Translocation of both H^+ and Na^+ occurs in methanogenic bioenergetics, so the cells can take advantage of the bifunctional ATP synthase without the necessity to utilize Na^+/H^+ antiporters.

Aim of this work was to solve unanswered questions regarding electron transport in methanogenic archaea of the genera *Methanosarcina* and *Methanosaeta*. The role and identity of ferredoxin oxidizing enzymes will be of particular interest, which will give a more complete understanding of the different ferredoxin: heterodisulfide oxidoreductase systems in methanogenic archaea.

Chapter 2

Function of Ech hydrogenase in ferredoxin-dependent, membrane-bound electron transport in *Methanosarcina mazei*

Ms. *mazei* and *Ms. barkeri* contain the membrane-bound multisubunit [NiFe] hydrogenase Ech that was described as ferredoxin oxidizing and H_2 producing (Meuer *et al.*, 1999). It forms part of the ferredoxin: heterodisulfide oxidoreductase that is responsible for energy conservation primarily in aceticlastic methanogenesis, but in part also in methyltrophic methanogenesis. In this paper a detailed analysis of a *Ms. mazei* Δ*ech* mutant is presented that complements the findings about a *Ms. barkeri* Δ*ech* mutant (Meuer *et al.*, 2002). Former works focused mainly on subunit composition, purification and role of Ech hydrogenase in biosynthetic pathways, which means the supply of reduced ferredoxin by H_2 oxidation in anabolic CO_2 reduction. This paper, however, focuses on the function of Ech hydrogenase as part of the respiratory chain, and reconstructs the electron flow from reduced ferredoxin to the heterodisulfide using washed membrane preparations of the wildtype and the Δ*ech* mutant strain. Furthermore, the phenotypic characterization of the Δ*ech* mutant reveals that the consumption of methylated amines is much faster with a lower growth yield as compared to the wildtype, and that growth on acetate is no longer possible. Overall, the metabolism of the Δ*ech* mutant seems to be ineffective and supports the hypothesis that Ech hydrogenase is an ion translocating enzyme.

Reduced ferredoxin as electron donor for membrane-bound electron transport in *Methanosarcina mazei*

Cornelia Welte[1], Verena Kallnik[1], Marcel Grapp[2], Gunes Bender[3], Steve Ragsdale[3], Uwe Deppenmeier[1]

1 Institute of Microbiology and Biotechnology, University of Bonn, Meckenheimer Allee 168, 53115 Bonn, Germany

2 Zentrum für Kinderheilkunde und Jugendmedizin, University of Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany

3 Department of Biological Chemistry, University of Michigan Medical School, 1150 W. Medical Center Dr. Ann Arbor, MI 48109-0606, USA

Reduced ferredoxin is an intermediate in the methylotrophic and aceticlastic pathway of methanogenesis and donates electrons to membrane-integral proteins, which transfer electrons to the heterodisulfide reductase. A ferredoxin interaction has already been observed for the Ech hydrogenase. Here we present the detailed analysis of a *Ms. mazei* Δech mutant which shows decreased ferredoxin-dependent membrane-bound electron-transport activity, a slower growth rate and faster substrate consumption. Evidence is presented that a second protein whose identity is unknown oxidizes reduced ferredoxin indicating an involvement in methanogenesis.

INTRODUCTION

The aceticlastic pathway of methanogenesis creates approximately 70 % (11) of the biologically produced methane and is of great ecological importance, as methane is a potent greenhouse gas. Organisms using this pathway to convert acetate to methane exclusively belong to the genera *Methanosarcina* and *Methanosaeta*. The two carbon atoms of acetate have different fates in the pathway: The methyl moiety is converted to methane whereas the carbonyl moiety is further oxidized to CO_2; the electrons derived from this oxidation step are used to reduce ferredoxin (Fd) (6). During methanogenesis from methylated C_1-compounds (methanol and methylamines) one quarter of the methyl groups are oxidized to obtain electrons for the reduction of heterodisulfide (28). A key enzyme in the oxidative part of methylotrophic methanogenesis is the formylmethanofuran dehydrogenase, which oxidizes the intermediate formylmethanofuran to CO_2 (7). The electrons are transferred to Fd. It was suggested that reduced ferredoxin (Fd_{red}) donates electrons to the respiratory chain with the heterodisulfide (CoM-S-S-CoB) as terminal electron acceptor and the reaction being catalyzed by the Fd_{red}:CoM-S-S-CoB oxidoreductase system (7; 25). The direct membrane-bound electron acceptor for Fd_{red} is still a matter of debate: for the Ech hydrogenase, a reduced ferredoxin accepting:H_2 evolving activity has already been observed for *Ms. barkeri* (21), which implies the involvement of the H_2:CoM-S-S-CoB oxidoreductase system in electron transport (14). The direct electron flow from the Ech hydrogenase to the heterodisulfide reductase has not been shown to date (21; 22). In contrast to *Ms. barkeri*, *Ms. acetivorans* is lacking the Ech hydrogenase (12). It can nevertheless grow on acetate, which is why another complex present in this organism, the Rnf complex, is discussed to be involved in the aceticlastic pathway of methanogenesis as acceptor for Fd_{red} (8; 11; 19). The *Ms. mazei* genome, however, contains genes coding for the Ech hydrogenase, but is lacking the Rnf complex (5).

To investigate whether the Ech hydrogenase is the only means by which *Ms. mazei* channels electrons from Fd_{red} into the respiratory chain, a mutant lacking the Ech hydrogenase (*Ms. mazei* Δech) has been constructed. Electron transport experiments using Fd_{red} as electron donor and CoM-S-S-CoB as electron acceptor were conducted with the wildtype and the mutant membranes to gain deeper insight into the actual membrane-bound protein complexes that accept electrons from Fd_{red}. Furthermore, an in-depth characterization of growth and substrate consumption of the Δech mutant was performed resulting in insight into the *in vivo* role of Ech hydrogenase.

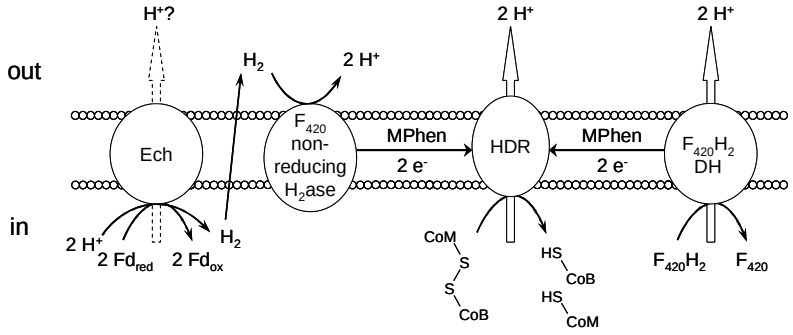

Figure 1: Proposed model of membrane-bound electron transfer in *Ms. mazei*. Proton translocation has been shown for the heterodisulfide reductase (15) and $F_{420}H_2$ dehydrogenase (2). Proton translocation for the Ech hydrogenase is assumed. H_2ase, hydrogenase; DH, dehydrogenase; HDR, heterodisulfide reductase; MPhen, methanophenazine. Fd, ferredoxin.

MATERIALS AND METHODS

Purification of proteins. Ferredoxin was purified from *Clostridium pasteurianum* DSM 525^T essentially as described by Mortenson (23). The last steps (dialyzation, crystallization) were replaced by ultrafiltration. Ferredoxin content was determined using the BCA method (27). CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) was purified from *Moorella thermoacetica* ATCC 39073 as described in (26) except for the following modifications. The extract was not heat treated or fractionated with ammonium sulfate. The first DEAE-cellulose column was eluted with a step gradient of 0.1 to 0.5 M NaCl, and CODH/ACS eluted at 0.3 M NaCl. CODH/ACS was then purified further using Q-sepharose anion exchange and phenyl-sepharose hydrophobic interaction columns, from which CODH/ACS eluted at ~0.4 M NaCl and ~0.2 M $(NH_4)_2SO_4$ respectively. The fractions from the phenyl-sepharose column that contained CODH/ACS were concentrated and buffer-exchanged into 50 mM Tris-HCl (pH 7.6) using Amicon ultra centrifuge concentrators in the anaerobic chamber (Vacum Atmospheres). Immediately after purification, the enzyme had a CO oxidation-methyl viologen reduction specific activity of 273 U/mg at 37 ºC.

Membrane preparations. *Methanosarcina mazei* DSM 7222 and derivatives were grown anaerobically at 37℃ in *Methanosarcina* medium (DSM medium 120) with trimethylamine as substrate (13). Subsequent steps were performed anaerobically in an anaerobic chamber (Coy laboratory products, US) under a 97 % N_2 / 3 % H_2 atmosphere. In the late exponential phase the cells were harvested and resuspended in phosphate buffer (40 mM KH_2PO_4/K_2HPO_4, pH 7.0, 5 mM DTE, 1 µg mL^{-1} resazurin) and incubated with DNAse I for 30 – 60 min at 4℃. As the buffer is hypoosmotic to the cells, they lyse immediately. The cell lysate was ultracentrifuged (1 h, 120,000 x g), the supernatant (cytoplasmic fraction) discarded and the membrane pellet homogenized in 20 mM phosphate buffer (KH_2PO_4/K_2HPO_4 pH 7.0) containing 20 mM $MgSO_4$, 500 mM sucrose, 5 mM DTE, 1 µg mL^{-1} resazurin. The ultracentrifugation step was repeated, the supernatant (which should be colourless) discarded and the membrane pellet again homogenized in the previously mentioned buffer. Protein content was determined using the Bradford method (3).

Enzyme assays. The photometrical tests were performed anaerobically in rubber stoppered 1.5 mL glass cuvettes in a V-550 UV/Vis spectrophotometer (Jasco, Germany). Benzylviologen-dependent heterodisulfide reductase activity was determined as a decrease of absorption at 575 nm using 600 µL phosphate buffer (40 mM KH_2PO_4/K_2HPO_4, pH 7.0, reduced with Ti(III) citrate), 625 nmol benzylviologen (ε_{575nm} = 8.9 mM^{-1} cm^{-1}), 300 nmol $Na_2S_2O_4$, 50 µg *Ms. mazei* membrane, 50 nmol CoM-S-S-CoB.

$F_{420}H_2$ dehydrogenase activity was determined as an increase of absorption at 420 nm using 600 µL phosphate buffer (40 mM KH_2PO_4/K_2HPO_4, pH 7.0, 5 mM DTE), 15 nmol $F_{420}H_2$ (ε_{420nm}=40 mM^{-1} cm^{-1}), 50 µg *Ms. mazei* membrane, 300 nmol metronidazole, 180 nmol methylviologen. F_{420} was isolated and reduced as described by (1). 1 U of activity was defined as 1 µmol electrons transported per min. CoM-S-S-CoB (synthesized according to (10; 24)) was quantified in anaerobic rubber stoppered glass vials containing 250 µL phosphate buffer (40 mM, 1 µg mL^{-1} resazurin, reduced with Ti(III) citrate) gassed with N_2/CO (5 % CO, purity 1.8, 95 % N_2, purity 5.0) for 1 min. For analysis of CoM-S-S-CoB reduction, 100 nmol CoM-S-S-CoB, 8.9 µg *Clostridium pasteurianum*

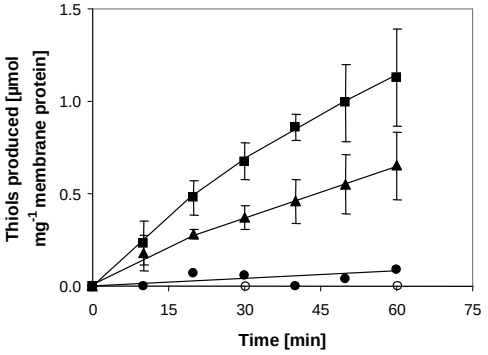

Figure 2: Fd-dependent heterodisulfide reduction with wildtype and Δech cytoplasmic membranes. The assays contained 100 nmol CoM-S-S-CoB, 8.86 µg Fd, 150 µg membrane and 75 µg CODH in a total volume of 250 µL. (■) wildtype membranes; (▲) membranes from Δech mutant; (●) control without Fd; (○) control without CO.

ferredoxin, 150 µg *Ms. mazei* membrane, 75 µg *Moorella thermoacetica* CO dehydrogenase/acetyl-CoA synthase were added. For CoM-SH/CoB-SH quantification (30) 20 µL samples were taken every 10 min for 1 h and directly used in a modified Ellman's assay: 950 µL Tris buffer (150 mM, pH 8.1) was mixed with the sample and 100 µL Ellman's reagent (5 mM 5,5'-dithiobis(2-nitrobenzoic acid) ($\varepsilon_{412\ nm}$=13.6 mM^{-1} cm^{-1}) in 50 mM sodium acetate buffer, pH 5.0), the absorption at 412 nm was immediately measured.

Alternatively, H_2:heterodisulfide oxidoreductase activity was determined by replacing CO, CO dehydrogenase/acetyl-CoA synthase and ferredoxin with H_2. Here, only 50 µg membrane preparation were used. 1 U of activity was defined as 1 µmol CoM-S-S-CoB reduced per min.

Generation of *Ms. mazei* Δech mutant. The *Ms. mazei* Δech mutant was generated by means of homologous recombination using the techniques described by Metcalf (20). Up- and downstream regions of the respective gene region were cloned into the two multiple cloning sites of pJK3 (20), linearized with *Apa*I and transformed into *Ms. mazei*. Instead of the respective gene, a puromycin resistance (*pac*) cassette was inserted. Puromycin resistant colonies were picked and screened for the knockout. Gene knockout was confirmed by sequencing and Southern blotting.

For construction of *Ms. mazei* Δech, the primers 5'-

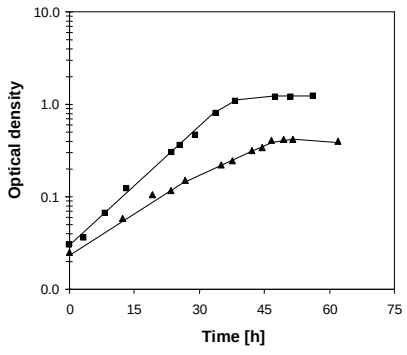

Figure 3: Growth curves for the wildtype (■) and Δech mutant (▲) during growth on TMA.

CCTACTCGAGGAGTGAATCAGCGAATAGAG-3' and 5'-GACCGAATTCACAACGTATCCTCCGACCTA-3' (upstream region of MM2320) with *Xho*I and *Eco*RI restriction sites and the primers 5'-CCAGGAGCTCGGTATGCTAAACCTTGTATT-3' and 5'-CAAGGGATCCCACTACAAATGTTGTCTCC-3' (MM2324 and downstream region) with *Sac*I and *Bam*HI restriction sites were used.

Growth analysis and substrate quantification. *Ms. mazei* wildtype and Δech mutant were grown as described above in 50 mL cultures with TMA as growth substrate. Optical density was measured at 600 nm on a Helios Epsilon Vis photometer (Thermo Scientific, Germany) in the presence of sodium dithionite. At different time points 700 µL samples were taken, cells and medium were separated by silicone oil centrifugation (100 µL silicone oil, 5 min, 14000 rpm) to thoroughly separate cells (which will lyse quickly after centrifugation as a result of aerobic conditions) from the medium.

TMA was quantified according to Ikawa (16): 100 µL sample were mixed with 20 µL trichloroacetic acid (10%), incubated for 5 min and then centrifuged for 5 min at 14000 rpm. 50 µL of this were mixed with 783 µL H_2O, 42 µL Folin-Ciocalteu reagent and 125 µL Na_2CO_3 (20%). After 30 min the absorbance at 745 nm was determined.

DMA was determined according to Dowden (9): 100 µL sample were mixed with 400 µL H_2O, 50 µL solution B (20 g ammonium acetate, 0.2 g $CuSO_4$ x 5 H_2O, 10 g NaOH, 20 mL NH_4OH, ad 100 mL H_2O), 500 µL solution A (5 % (v/v) carbon disulfide in benzene), 50 µL solution C (30 % acetic acid in H_2O). The vial was mixed thoroughly, centrifuged for 2 min at 15000 rpm and the absorbance at 430 nm of the upper organic layer was determined.

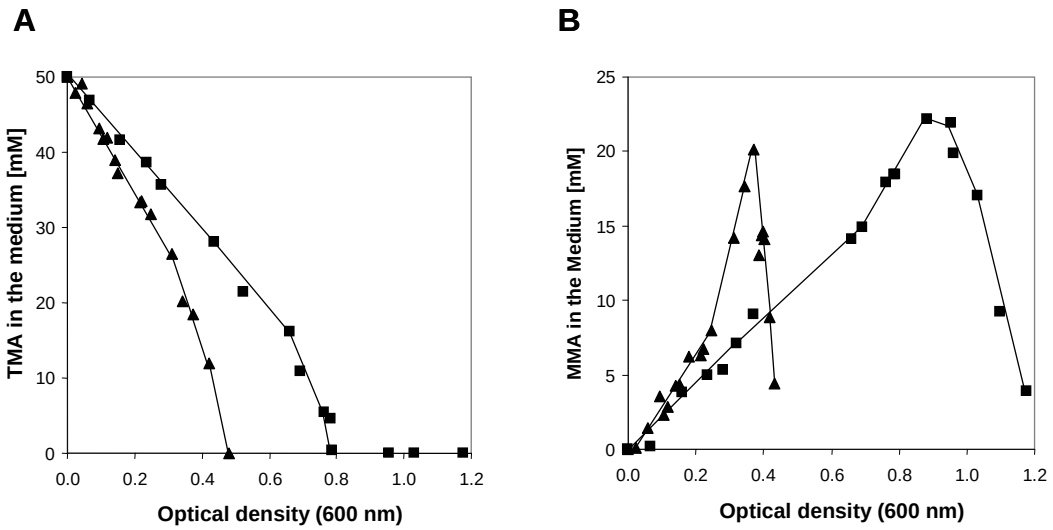

Figure 4: Substrate utilization of the wildtype (■) and the Δech mutant (▲) during growth with 50 mM TMA as substrate. (A) TMA consumption. (B) MMA production and consumption.

MMA was quantified according to Wang (29): 50 µL sample were mixed with 200 µL sodium acetate (100 mM, pH 5.6), 300 µL acetylacetone/formaldehyde solution (4 mL acetylacetone, 10 mL formaldehyde (30 %), 136 mL H_2O), 450 µL acetone. This mixture was boiled at 95°C for 5 min, then directly put on ice , and the absorbance at 410 nm immediately determined.

TMA, DMA and MMA contents were measured as triplicates and compared to standard curves.

RESULTS AND DISCUSSION

To investigate ferredoxin-mediated membrane-bound electron transport in *Ms. mazei*, an *in vitro* assay with washed membrane preparations of the *Ms. mazei* wildtype and Δech mutant strain grown on trimethylamine was established. In this assay, electrons were transferred from the initial substrate CO to the *Moorella thermoacetica* CO dehydrogenase/acetyl-CoA synthase (CODH/ACS), which reduces *Clostridium pasteurianum* ferredoxin (Fd). Fd donates electrons to different membrane-bound complexes (described in detail below), which are predicted to reduce methanophenazine (1). Methanophenazine is the electron donor for the cytochrome *b* subunit of the heterodisulfide reductase (Fig. 1), which reduces the terminal electron acceptor CoM-S-S-CoB (4). The activity of the Fd_{red}:heterodisulfide oxidoreductase system was about 24 mU/mg membrane protein (eq. 1) and was confirmed with three independent membrane preparations obtained from different *Ms. mazei* cultures (Fig. 2).

(eq. 1) $2\ Fd_{red} + CoM\text{-}S\text{-}S\text{-}CoM + 2\ H^+ \rightarrow 2\ Fd_{ox} + HS\text{-}CoM + HS\text{-}CoB$

This activity was similar to the values obtained by Peer (25) who measured an activity of 23 mU mg^{-1} for the *Ms. thermophila* CO: CoM-S-S-CoB oxidoreductase system. The apparent requirement for Fd in the *Ms. mazei* system makes it a suitable tool to investigate Fd-dependent reduction of heterodisulfide. CoM-S-S-CoB reduction was clearly dependent on the presence of CO, CODH/ACS, Fd and membranes. If one component was omitted, heterodisulfide reducing activity could not be measured (Fig. 2). Chemical degradation of heterodisulfide could not be observed in the reaction mixtures.

According to the current model (Fig. 1), Fd_{red} produced in the aceticlastic as well as in the methylotrophic pathway is oxidized by the Ech hydrogenase, which releases molecular hydrogen (21). The Ech hydrogenase is membrane-bound and consists of six subunits (EchA – EchF). Hydrogen produced in the first reaction is re-oxidized by the F_{420} nonreducing hydrogenase (Fig. 1) and electrons are subsequently shuttled to the heterodisulfide reductase, which reduces CoM-S-S-CoB (15). In *Ms. barkeri*, Fd_{red} oxidizing:H_2 evolving activities have already been observed for the purified Ech hydrogenase which underlines the above presented hypothesis (21). The coupling of Ech hydrogenase activity and CoM-S-S-CoB reduction, however, has not been shown to date. To analyze this process in more detail, we constructed a *Ms. mazei* Δech mutant and subjected the membrane fraction to the CoM-S-S-CoB reduction assays. The activity of *Ms. mazei* Δech mutant membrane fraction

decreased by about 50 % compared to the wildtype (Fig. 2). This decrease in activity confirms that in *Ms. mazei* the Ech hydrogenase also accepts electrons from Fd_{red}. However, the Fd_{red} oxidizing activity was not completely abolished in the *Ms. mazei* Δech mutant and reached half of the activity of the wildtype. Hence, it is tempting to speculate that there is at least one membrane-bound protein that is different from the Ech hydrogenase and accepts electrons from Fd_{red}, allowing the cell to gain energy by electron transport phosphorylation during aceticlastic as well as methylotrophic growth. In *Ms. acetivorans*, genes for the Ech hydrogenase are absent, but the organism can nevertheless grow on acetate where Fd_{red} oxidation by a membrane-bound protein complex is crucial. Proteome analysis suggested that another membrane-bound complex referred to as Rnf is involved in acetate metabolism and hence in the respiratory chain and the oxidation of Fd_{red} (18; 19). In contrast, *Ms. mazei* posseses an Ech hydrogenase but the genomic data indicate that the Rnf complex is absent.

To assure the correct functioning of the Fd-independent parts of the respiratory chain (Fig. 1), H_2:heterodisulfide reductase activities, benzylviologen dependent heterodisulfide reductase assays and $F_{420}H_2$ dehydrogenase activity assays were performed with wildtype and the deletion mutant membrane preparations. The H_2:heterodisulfide reductase exhibited an activity of approximately 150 mU mg^{-1} protein in the wildtype and the ech mutant, indicating a 5-fold increase compared to the CO-system. The higher activity of the H_2:CoM-S-S-CoB oxidoreductase system probably results from a limited interaction of the non-native reaction components with the Ech hydrogenase in our test system. Also the benzylviologen-dependent heterodisulfide reductase and the $F_{420}H_2$ dehydrogenase activities were similar for all *Ms. mazei* membranes tested. Hence, the deletion of the Ech hydrogenase had no influence on the H_2 and $F_{420}H_2$-dependent electron transport systems in *Ms. mazei* (Fig. 1).

The *Ms. mazei* Δech mutant did not grow on acetate as already observed by Meuer (22) for the *Ms. barkeri* Δech mutant. When trimethylamine (TMA) was used as growth substrate, distinct differences in growing behavior could be observed. TMA is demethylated step-wise to dimethylamine (DMA) and monomethylamine (MMA), which are in part excreted into the growth medium. DMA is found only as an intermediate product with very low concentrations in the culture supernatant, whereas MMA accumulates and is only used when TMA and DMA are completely consumed (17). The respective methyl groups are transferred to CoM via substrate-specific methyl transferases to yield methyl-CoM (17). Three quarters of the methyl moieties are reduced to CH_4 leading to the formation of heterodisulfide (CoM-S-S-CoB; Fig. 1). One quarter of methyl-CoM is oxidized to CO_2 to yield reducing equivalents or electrons that are transferred to F_{420} and ferredoxin, respectively (28; Fig. 1). $F_{420}H_2$ is oxidized by the membrane-bound $F_{420}H_2$ dehydrogenase (Fig. 1), whereas Fd_{red} is recycled by the Fd_{red}:heterodisulfide oxidoreductase system, which is Ech-independent in the Δech mutant.

When growth of the *Ms. mazei* wildtype and Δech mutant with TMA as substrate were compared, apparent doubling times of 7.7 h for the wildtype and 9.1 h for the Δech mutant were observed. Furthermore, the wildtype grew up to an OD_{600} of 1.2 whereas the Δech mutant stopped growing at an OD_{600} of around 0.5 (Fig. 3). The TMA consumption rate of the Δech mutant was increased 2-fold compared to the wildtype; TMA was completely consumed at an OD_{600} of 0.48 in the Δech mutant and 0.80 for the wildtype (Fig. 4A). As mentioned previously, MMA accumulates in the culture supernatant as a product from TMA and DMA breakdown and is only used when TMA and DMA are completely metabolized. Thus, MMA concentration in the culture supernatant first increases to a certain amount and then decreases again. The MMA formation rate increased 2.7-fold in the Δech mutant compared to the wildtype. The MMA degradation rate showed the same tendency, it increased 2.5-fold compared to the wildtype (Fig. 4B).

In summary, the electron transport deficiency of the Δech mutant is reflected in its growth abilities as it grows slower but uses more substrate to generate less biomass. Taken together, these data argue for a deficient energy coupling site in the Δech mutant, thus a H^+ or Na^+ translocating activity of the Ech hydrogenase as proposed (but not demonstrated) by Meuer (21) is very likely.

Overall the results indicate that the ferredoxin-mediated membrane-bound electron transport chain is more complex than previously thought, and that the Ech hydrogenase as well as another not yet identified membrane-bound protein is involved in aceticlastic and methylotrophic methanogenesis of *Ms. mazei*.

Acknowledgements

We thank Elisabeth Schwab for technical assistance and Paul Schweiger for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (grant De488/9-1).

REFERENCES

1. **Abken, H. J., M. Tietze, J. Brodersen, S. Bäumer, U. Beifuss, and U. Deppenmeier**. 1998. Isolation and characterization of methanophenazine and the function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Gö1. J. Bacteriol. **180**: 2027-2032.

2. **Bäumer, S., T. Ide, C. Jacobi, A. Johann, G. Gottschalk, and U. Deppenmeier**. 2000. The $F_{420}H_2$ dehydrogenase from *Methanosarcina mazei* is a redox-driven proton pump closely related to NADH dehydrogenases. J. Biol. Chem. **275**: 17968-17973.

3. **Bradford M. M.**. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

4. **Brodersen, J., G. Gottschalk, and U. Deppenmeier**. 1999. Membrane-bound $F_{420}H_2$-dependent heterodisulfide reduction in *Methanococcus voltae*. Arch. Microbiol. **171**: 115-121.

5. **Deppenmeier, U., A. Johann, T. Hartsch, R. Merkl, R. A. Schmitz, R. Martinez-Arias, A. Henne, A. Wiezer, S. Bäumer, C. Jacobi, H. Brüggemann, T. Lienard, A. Christmann, M. Bömeke, S. Steckel, A. Bhattacharyya, A. Lykidis, R. Overbeek, H. P. Klenk, R. P. Gunsalus, H. J. Fritz, and G. Gottschalk**. 2002. The genome of *Methanosarcina mazei*: evidence for lateral gene transfer between bacteria and archaea. J. Mol. Microbiol. Biotechnol. **4**: 453-461.

6. **Deppenmeier, U., and V. Müller**. 2008. Life close to the thermodynamic limit: How methanogenic archaea conserve energy. Results Probl. Cell Differ. **45**: 123-152.

7. **Deppenmeier, U.**. 2002. The unique biochemistry of methanogenesis. Prog. Nucl. Acid Res. Mol. Biol. **71**: 223-283.

8. **Ding, Y. H., and J. G. Ferry**. 2004. Flavin mononucleotide-binding flavoprotein family in the domain Archaea. J. Bacteriol. **186**: 90-97.

9. **Dowden, H. C.**. 1938. The determination of small amounts of dimethylamine in biological fluids. Biochem. J. **32**: 455–459.

10. **Ellermann, J., A., Kobelt, A. Pfaltz, and R. K. Thauer**. 1987. On the role of N-7-mercaptoheptanoyl-O-phospho-L-threonine (component B) in the enzymatic reduction of methyl-coenzyme M to methane. FEBS letters **220**: 358-362.

11. **Ferry, J.G., and D. J. Lessner**. 2008. Methanogenesis in marine sediments. Ann. N.Y. Acad. Sci. **1125**:147-157.

12. **Galagan, J. E. et al.**. 2002. The genome of *Methanosarcina acetivorans* reveals extensive metabolic and physiological diversity. Genome Res. **12**: 532-542.

13. **Hippe, H., D. Caspari, K. Fiebig, and G. Gottschalk**. 1979. Utilization of trimethylamine and other N-methyl compounds for growth and methane formation by *Methanosarcina barkeri.* Proc. Natl. Acad. Sci. USA **76**: 494-498.

14**. Hovey, R., S. Lentes, A. Ehrenreich, K. Salmon, G. Gottschalk, R. P. Gunsalus, and U. Deppenmeier**. 2005. DNA microarray analysis of *Methanosarcina mazei* during growth on methanol and acetate. Mol. Genet. Genomics **273**: 225-239.

15. **Ide T., S. Bäumer, and U. Deppenmeier**. 1999. Energy conservation by the H_2:heterodisulfide oxidoreductase from *Methanosarcina mazei* Gö1: Identification of two proton-translocating segments. J. Bacteriol. **181**: 4076-4080.

16. **Ikawa, M., T. D. Schaper, C. A. Dollard, and J. J. Sasner**. 2003. Utilization of the Folin-Ciocalteu phenol reagent for the determination of TMA. J. Agric. Food Chem. **51**: 1811-1815.

17. **Krätzer, C., P. Carini, R. Hovey, and U. Deppenmeier**. 2009. Transcriptional profiling of methyltransferase genes during growth of *Methanosarcina mazei* on trimethylamine. J. Bacteriol. **191**: 5108-5115.

18. **Lessner, D. J., and J. G. Ferry**. 2007. The archaeon *Methanosarcina acetivorans* contains a protein disulfide reductase with an iron-sulfur cluster. J. Bacteriol. **189**: 7475-7484.

19. **Li, L., Q. Li, L. Rohlin, U. Kim, K. Salmon, T. Rejtar, R. P. Gunsalus, B. L. Karger, and J. G. Ferry**. 2007. Quantitative proteomic and microarray analysis of the archaeon *Methanosarcina acetivorans* grown with acetate versus methanol. J. Proteome Res. **6**: 759-771.

20. **Metcalf, W. W., J. K. Zhang, E. Apolinario, K. R. Sowers, and R. S. Wolfe**. 1997. A genetic system for Archaea of the genus *Methanosarcina*: liposome-mediated transformation and construction of shuttle vectors. Proc. Natl. Acad. Sci. USA **94**: 2626-2631.

21. **Meuer, J., S. Bartoschek, J. Koch, A. Künkel, and R. Hedderich**. 1999. Purification and catalytic properties of Ech hydrogenase from

Methanosarcina barkeri. Eur. J. Biochem. **265**: 325-335.

22. **Meuer, J., H. C. Kuettner, J. K. Zhang, R. Hedderich, and W. W. Metcalf**. 2002. Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. Proc. Natl. Acad. Sci. USA **99**: 5632-5637.

23. **Mortenson, L. E.**. 1964. Purification and analysis of ferredoxin from *Clostridium pasteurianum*. Biochim. Biophys. Acta **81**: 71-77.

24. **Noll, K. M., K. L. Rinehart Jr., R. S. Tanner, and R. S. Wolfe**. 1986. Structure of component B (7-mercaptoheptanoylthreonine phosphate) of the methylcoenzyme M methylreductase system of *Methanobacterium thermoautotrophicum*. Proc. Natl. Acad. Sci. USA **83**: 4238-4242.

25. **Peer, C.W., M. H. Painter, M. E. Rasche, and J. G. Ferry**. 1994. Characterization of a CO: heterodisulfide oxidoreductase system from acetate-grown *Methanosarcina thermophila*. J. Bacteriol. **176**: 6974-6979.

26. **Ragsdale S. W., L. G. Ljungdahl, and D. V. Der Vartanian**. 1983. Isolation of carbon monoxide dehydrogenase from *Acetobacterium woodii* and comparison of its properties with those of the *Clostridium thermoaceticum* enzyme. J. Bacteriol. **155**: 1224-1237.

27. **Smith, P.K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk**. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**: 76-85.

28. **Thauer, R.K., A. K. Kaster, H. Seedorf, W. Buckel, and R. Hedderich**. 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. **6**: 579-591.

29. **Wang, H. Y., L. D. Liua, and J. C. Renb**. 1999. Determination of methyl isocyanate in air by fluorimetry. Analyst **124**:1327–1330.

30. **Zahler, W.L. and W. W. Cleland**. 1968. A specific and sensitive assay for disulfides. J. Biol. Chem. **243**: 716-719.

Chapter 3

Involvement of Ech hydrogenase in energy conservation of *Methanosarcina mazei*

Under aceticlastic growth conditions, ferredoxin is the only reducing equivalent that is produced in the central methanogenic pathway, and the ferredoxin: heterodisulfide oxidoreductase is the only respiratory system by which the proton motive force is generated. In *Ms. mazei* and *Ms. barkeri*, Ech hydrogenase is a part of the ferredoxin: heterodisulfide oxidoreductase, and catalyzes the oxidation of ferredoxin with concomitant H_2 production. The H_2 is consumed by the H_2: heterodisulfide oxidoreductase system that translocates 4 H^+ / 2 e^- across the cytoplasmic membrane yielding about one ATP equivalent. One ATP is also consumed in the initial activation reaction of acetate breakdown. The net energy gain consists of 1-2 translocated Na^+ ions in the H_4SPT: CoM methyl transferase reaction (Becher *et al.*, 1992, Lienard *et al.*, 1996). Cell lysate experiments and comparative genomics indicated that the reaction catalyzed by Ech hydrogenase could also be energy conserving. The in-depth investigation of the *Ms. mazei* Δ*ech* mutant in the previous chapter supports this hypothesis. This paper focuses on the function of the *Ms. mazei* Ech hydrogenase in energy conservation. A vesicular system of cytoplasmic membranes revealed that during the Ech hydrogenase reaction an ion gradient is built that is evidently used for ATP synthesis. Methanogenic bioenergetics harbours possibilities for Na^+ translocation (H_4SPT: CoM methyl transferase) and H^+ translocation (F_{420} non-reducing hydrogenase, heterodisulfide reductase, $F_{420}H_2$ dehydrogenase), and the nature of the ion translocated by Ech hydrogenase had to be determined. Inhibitor studies identified the translocated ion as a proton, and theoretical considerations led to the stoichiometry of 1 H^+ / 2 e^-.

Welte *et al.* (2010) *FEBS J* **277**:3396-3403

Involvement of Ech hydrogenase in energy conservation of *Methanosarcina mazei*

Cornelia Welte, Christian Krätzer, Uwe Deppenmeier

Institute of Microbiology and Biotechnology, University of Bonn, Meckenheimer Allee 168, 53115 Bonn, Germany

Methanosarcina mazei **belongs to the group of aceticlastic methanogens and converts acetate into the potent greenhouse gases CO_2 and CH_4. The aceticlastic respiratory chain involved in methane formation comprises the three transmembrane proteins Ech hydrogenase, F_{420} non-reducing hydrogenase and heterodisulfide reductase. It was shown that the latter two contribute to the proton motive force. The data presented here clearly demonstrate that the Ech hydrogenase is also involved in energy conservation. ATP synthesis was observed in a cytoplasm-free vesicular system of *Methanosarcina mazei* that was dependent on the oxidation of reduced ferredoxin and the formation of molecular hydrogen (as catalyzed by Ech hydrogenase). Such an ATP formation was not observed in a Δ*ech* mutant strain. The protonophore SF6847 led to complete inhibition of ATP formation in the *Ms. mazei* wildtype without inhibiting hydrogen production by Ech hydrogenase, whereas the sodium ion ionophore ETH157 did not affect ATP formation in this system. Thus, we conclude that Ech hydrogenase acts as primary proton pump in a ferredoxin-dependent electron transport system.**

INTRODUCTION

Biological methanogenesis from acetate is one of the most important processes for the maintenance of the carbon cycle on earth. The products of methanogenesis from acetate, CH_4 and CO_2, are released from anaerobic habitats and large amounts of these greenhouse gases reach the atmosphere. Therefore, the process of biological methane formation is of great interest for global ecology [1, 2]. Moreover, the process of methanogenesis creates a combustible gas that can be used as an energy source. Only the genera *Methanosarcina* and *Methanosaeta* are able to use the aceticlastic pathway of methanogenesis, and *Methanosarcina mazei* strain Gö1 (from here referred to as *Ms. mazei*) is one of the important model organisms [3]. In *Ms. mazei*, acetate is activated by phosphorylation and exchange of inorganic phosphate with coenzyme A (CoA). The resulting acetyl-CoA is cleaved by the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). In the course of the reaction enzyme-bound CO is oxidized to CO_2 and the electrons are used for ferredoxin (Fd) reduction. The methyl group of acetate is transferred to tetrahydrosarcinapterin (H_4SPT). The resulting methyl-H_4SPT is converted to methane by the catalytic activities of a Na^+-translocating methyl-CoM methyltransferase (forming methyl-2-mercaptoethanesulfonate (methyl-S-CoM)) and the methyl-S-CoM reductase, which uses N-7-mercaptoheptanoyl-L-threonine phosphate (HS-CoB) as the electron donor to reduce the methyl group to CH_4. An additional product of this reaction is the heterodisulfide of HS-CoM and HS-CoB (CoM-S-S-CoB), which serves as terminal electron acceptor in the methanogenic respiratory chain (for review see [4]).

The intermediates of the aceticlastic pathway, CoM-S-S-CoB and reduced ferredoxin (Fd_{red}), are recycled by a membrane-bound electron transport system which can be defined as ferredoxin:heterodisulfide oxidoreductase [5]. In most *Methanosarcina* species (e.g. *Ms. mazei* and *Ms. barkeri*) the oxidation of reduced ferredoxin is catalyzed by Ech hydrogenase resulting in the release of molecular hydrogen [6] that is then reoxidized by the F_{420} non-reducing hydrogenase and channelled via methanophenazine to the heterodisulfide reductase [7]. Some *Methanosarcina* species, e.g. *Ms. acetivorans*, lack Ech hydrogenase and must possess an alternative route for oxidation of Fd_{red}. It was shown that the F_{420} non-reducing hydrogenase and the heterodisulfide reductase are key elements in membrane-bound electron transport and are essential to generate the proton motive force [7], whereas the methyl-CoM methyltransferase generates a Na^+ ion

Table 1: Hydrogen formation by reduced ferredoxin-dependent proton reduction. Test vials contained 5 % CO / 95 % N_2 in the headspace, 500 µg inverted membrane vesicles, 33.5 µg ferredoxin, 20 µg CODH/ACS, 150 nmol AMP, 300 nmol ADP. Addition or exclusion of single components is indicated.

Preparation	Assay condition	H_2 production rate (%)
wt[1] vesicles	complete	100[2]
wt vesicles	+ 10 µM ETH157	101
wt vesicles	+ 10 µM SF6847	130
wt vesicles	+ 400 µM DCCD	99
wt vesicles	without Fd	< 1
wt vesicles	without CO	< 1
Δech vesicles	complete	< 1

1) wildtype
2) most active vesicle preparation showed a specific activity of 32.8 nmol min^{-1} x mg $protein^{-1}$

gradient [8, 9]. Furthermore, it was suggested that the Ech hydrogenase also contributes to the electro chemical ion gradient [5] because of homologies to certain subunits of ion-translocating oxidoreductases [10] and indirect evidence from experiments with resting cells of *Ms. barkeri* [11, 12]. However, direct experimental evidence for this hypothesis is lacking. In this study, we present the first biochemical proof that Ech hydrogenase is indeed an ion-translocating enzyme, and thus represents an additional energy conserving coupling site in methanogenic metabolism. Inhibitor studies clearly indicate that H^+ and not Na^+ is the coupling ion, thus the proton gradient can directly be used for ATP synthesis via A_1A_O ATP synthase [13].

RESULTS

To investigate ferredoxin-mediated electron transport, we took advantage of washed inverted vesicle preparations of *Ms. mazei*, which contain all essential membrane-bound proteins involved in energy conservation and which are suitable for the generation of electro-chemical ion gradients [14]. These vesicles do not contain enzymatic activities that would produce Fd_{red}. Therefore, Fd from *Clostridum pasteurianum* was used as electron donor, which was reduced by the CODH/ACS from *Moorella thermoacetica* with CO as initial substrate.

When the oxidation of Fd_{red} in the absence of CoM-S-S-CoB was analyzed in the washed vesicle preparation the rate of H_2 production was 32.8 nmol x min^{-1} x mg $protein^{-1}$ (Tab. 1) and was constant over a time period of 60 min. The reaction was coupled to the phosphorylation of ADP as indicated by a rapid increase of the ATP content upon the start of the reaction (Fig. 1). The rate of ATP production was 1.5 nmol ATP min^{-1} x mg $protein^{-1}$, which is comparable to ATP synthesis rates observed in the process of methanogenesis from methyl-S-CoM + H_2 [15]. In the absence of Fd or CO, H_2 production was < 0.1 nmol x min^{-1} x mg $protein^{-1}$ (Tab. 1) and ATP synthesis was not observed (Fig. 1). However, ATP synthesis (Fig. 1) and H_2 formation (not shown) were fully restored when Fd was subsequently added to the reaction mixture.

To analyze this process in more detail, we used washed vesicle preparations of a *Ms. mazei* Δ*ech* mutant and subjected these vesicles to the standard assay (described in Materials and Methods under "Determination of ATP formation"). As expected, H_2 formation from Fd_{red} was not observed in this mutant (Tab. 1), whereas the activities of all other Fd-independent parts of the respiratory chain (F_{420} non-reducing hydrogenase, heterodisulfide reductase and $F_{420}H_2$ dehydrogenase) remained unaffected (not shown). As evident from Fig. 2 inverted membrane vesicles from the Δ*ech* mutant did not form ATP when incubated with reduced ferredoxin in the absence of heterodisulfide. As a control, ATP formation associated with H_2:heterodisulfide oxidoreductase activity was examined and the rate of ATP formation (1.9 nmol ATP min^{-1} x mg $protein^{-1}$) in vesicle preparations was the same for the mutant and the wildtype with H_2 and CoM-S-S-CoB as substrates (Fig. 2). This process was independent of Ech hydrogenase because the F_{420} non-reducing hydrogenase oxidizes H_2 and electrons are transferred via methanophenazine to heterodisulfide reductase (Hdr). This process is coupled to proton translocation over the cytoplasmic membrane [7]. In summary, these results clearly indicated that the Ech

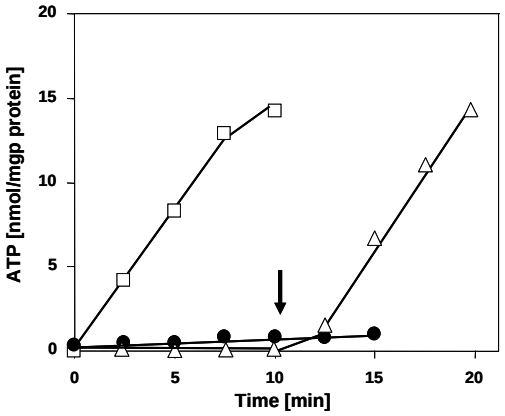

Figure 1: Ferredoxin-dependent ATP synthesis. Test vials contained 5 % CO / 95 % N_2 in the headspace, 500-700 µg inverted membrane vesicles, 33.5 µg ferredoxin, 20 µg CODH/ACS, 150 nmol AMP, 300 nmol ADP. (□) positive control; (Δ) control without ferredoxin, arrow indicates addition of 33.5 µg Fd; (●) control without CO.

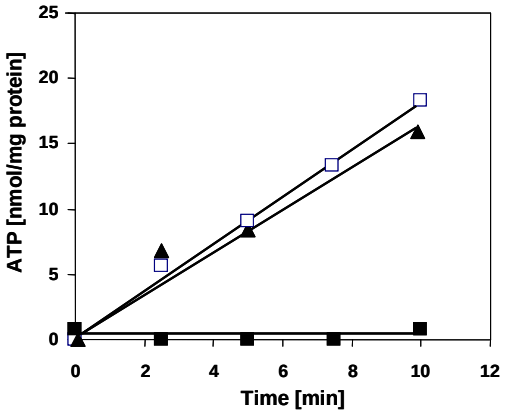

Figure 2: ATP synthesis by wildtype and Δ*ech* mutant. Test vials contained 500-700 µg inverted membrane vesicles, 150 nmol AMP, 300 nmol ADP. (■) 5 % CO / 95 % N_2 in the headspace, 33.5 µg ferredoxin, 20 µg CODH/ACS, Δ*ech* mutant vesicle preparation; (□) 100 % H_2 in the headspace, 150 nmol CoM-S-S-CoB, Δ*ech* mutant vesicle preparation; (▲) 100 % H_2 in the headspace, 150 nmol CoM-S-S-CoB, wildtype vesicle preparation .

hydrogenase is necessary to generate an electrochemical ion gradient when ferredoxin is the only reducing equivalent and heterodisulfide is absent.

To rule out the possibility of substrate-level phosphorylation, independent of an ion gradient, *N,N*'-dicyclo-hexylcarbodiimide (DCCD) was added to the reaction. This compound specifically inhibits the ATP synthase from *Ms. mazei* [13], and 400 µM DCCD fully inhibited ATP synthesis (Fig. 3), whereas H_2 evolution, as indicator for Ech hydrogenase activity, was not affected (Tab. 1). Taken together, these data indicated that energy is conserved by the Ech hydrogenase by the generation of an ion gradient and ATP synthesis by the catalytic activity of the A_1A_O ATP synthase.

However, the nature of the ion translocated over the cytoplasmic membrane still remained unclear. Protons and sodium ions are proposed as coupling ions [5], but biochemical evidence for either is missing. Therefore, inhibitor studies were performed to identify the translocated ion. It was already shown that the Na^+ ionophore ETH157 effectively dissipates Na^+ gradients in vesicular systems of *Ms. mazei* [8]. As evident from Fig. 3 addition of ETH157 did not show any effect on the rate of ATP synthesis in the washed membrane vesicle system or on H_2 formation (Tab. 1), indicating that Na^+ is not the coupling ion of Ech hydrogenase. In contrast, 10 µM 3,5-di-tert-butyl-4-hydroxybenzylidene-malononitrile (SF6847), a potent protonophore [16], fully inhibited Fd_{red}-dependent ATP formation. To ensure that SF6847 only abolished the formation of an H^+ gradient used for ATP synthesis and not Ech hydrogenase activity, H_2 evolution rates were measured (Tab. 1). Samples containing 10 µM of the protonophore SF6847 exhibited H_2 evolving rates of 42 nmol H_2 min^{-1} x mg $protein^{-1}$ and were higher than the control assay without the uncoupler. The effect of SF6847 on the rate of electron transport resembles the phenomenon of respiratory control that was observed before in the *Ms. mazei* vesicle system when ATP synthesis was analyzed by proton translocation coupled to the H_2:heterodisulfide oxidroreductase system [16].

DISCUSSION

The energy conserving transmembrane enzyme system used in the aceticlastic pathway of methanogenesis has been referred to as ferredoxin:heterodisulfide oxidoreductase. The electron flow from Fd_{red} to heterodisulfide reductase in *Ms. mazei* could be reconstructed in recent years (Fig. 4). Fd_{red} is oxidized by Ech hydrogenase that produces H_2 by proton reduction [6]. The F_{420} non-reducing hydrogenase oxidizes H_2 on the outside of the cytoplasmic membrane [7], thereby releasing two protons. The electrons and 2 H^+ from the cytoplasm are used for the reduction of methanophenazine, which is a membrane-

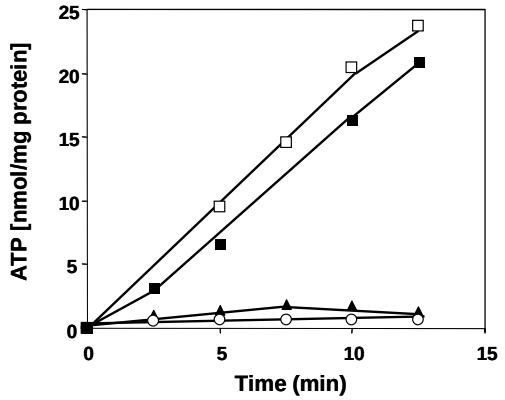

Figure 3: Influence of inhibitors on ATP synthesis. Assay conditions as in Fig. 1. (□) positive control without ionophore; (■) 10 µM ETH157; (○) 10 µM SF6847; (▲) 400 µM DCCD.

integral electron carrier in *Methanosarcina* species [17]. Reduced methanophenazine transfers electrons to heterodisulfide reductase (Fig. 4). The respective protons are released into the extracellular space [7] thereby generating an electro-chemical proton gradient, which is used for ATP synthesis by the A_1A_O ATP synthase. Energy conservation for Ech hydrogenase based on growth data and experiments on resting cells and cell suspensions has been proposed in several studies [6, 12, 18-20], but ATP production or generation of an H^+ or Na^+ gradient directly by Ech hydrogenase has not been reported. The data presented here clearly demonstrate a direct involvement of Ech hydrogenase in energy conservation: i) ATP synthesis was observed in the *Ms. mazei* vesicular system that was dependent on the oxidation of Fd_{red} (catalyzed by Ech hydrogenase). ii) The *Ms. mazei* Δ*ech* mutant showed no formation of ATP in the presence of Fd_{red}. In contrast, ATP synthesis from H_2 + CoM-S-S-CoB was identical to wildtype levels, indicating that the Δ*ech* vesicle preparation was able to establish an ion gradient and that the ATP synthase was active. iii) Addition of protonophore SF6847 led to complete cessation of ATP formation without inhibiting the Ech hydrogenase, whereas the sodium ion ionophore ETH157 did not affect ATP formation in this system. Therefore, protons are clearly used as coupling ions.

Proton translocation by Ech hydrogenase is similar to studies performed on the related Mbh hydrogenase from *Pyrococcus furiosus* [21], which also translocates protons in the process of Fd_{red} oxidation. Both proteins belong to a small subset of multi-subunit [NiFe] hydrogenases within the large group of [NiFe] hydrogenases, that use reduced ferredoxin or polyferredoxin as an electron donor [10]. Members of this group are thought to couple hydrogen formation to energy conservation, primarily based on their homology to the proton pumping NADH:ubiquinone oxidoreductase (complex I). Biochemical evidence of proton translocation has so far only been presented for the Mbh [NiFe] hydrogenase from *P. furiosus* [21]. Other members of this group are the Coo [NiFe] hydrogenases from *Rhodospirillum rubrum* [22] and *Carboxydothermus hydrogenoformans* [23], and the Hyc and Hyf [NiFe] hydrogenases from *Escherichia coli* [24-26]. Ech hydrogenase is now another member of the group of energy-conserving multi-subunit [NiFe] hydrogenases to that an energy conserving function can be assigned due to biochemical data and not solely based on sequence similarity to complex I or Mbh hydrogenase of *P. furiosus*.

It is evident that the proton gradients generated by the Ech hydrogenase from *Ms. mazei* and the Mbh hydrogenase from *Pyrococcus furiosus* is used for ATP synthesis catalysed by A_1A_o-type ATP synthases. It was shown [27] that the enzyme from *Ms. mazei* has high sequence similarities to the Na^+ translocating A_1A_o ATPase from *P. furiosus*, but experimental data clearly show that the enzyme is H^+-dependent. In contrast, the ATP synthase from *Pyrococcus furiosus* uses the sodium ion gradient for ATP synthesis [28]. Directly adjacent to the Mbh hydrogenase a gene encoding a Na^+/H^+ antiporter was found. Hence, the electrochemical proton gradient across the cytoplasmic membrane could be converted to a sodium ion potential by action of the Na^+/H^+ antiporter.

Under standard conditions the CO dependent H_2 evolution is coupled to a change of free energy of –19.3 kJ/mol ($\Delta E^{0'}$= 0.1 V). According to the equation $n = 2\Delta E_h/\Delta p$ (with n = number of translocated protons, ΔE_h = redox potential difference, Δp = electrochemical potential which is about 0.15 V in methanogens [29]) the Ech hydrogenase is able to translocate about one proton per hydrogen molecule formed. In many living cells three protons are needed for the phosphorylation of ADP as catalyzed by ATP synthases [30]. Assuming that the Ech hydrogenase translocates one proton per hydrogen molecule, the ratio of ATP synthesis and H_2 production should be in the range of 0.33. The results presented showed rates of 1.5 nmol ATP x min^{-1} x mg^{-1} and 32.8 nmol H_2 x min^{-1} x mg^{-1} resulting in a ATP/H_2 stoichiometry of 0.05

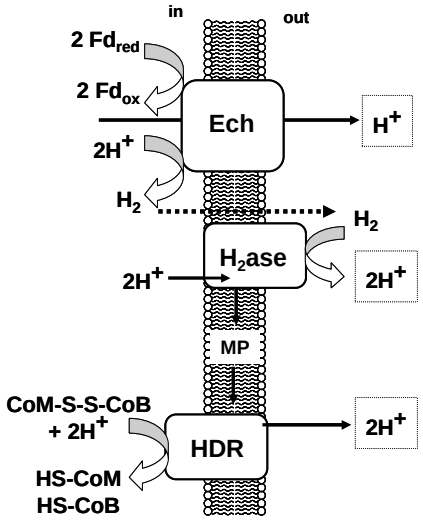

Figure 4: Proposed model of ferredoxin-dependent electron transport chain in *Ms. mazei*. H_2ase, hydrogenase; HDR, heterodisulfide reductase; MP, methanophenazine. Fd, ferredoxin.

in the vesicular system of *Ms. mazei*. The apparent discrepancy is most probably due to disintegrated membrane vesicles in the vesicle preparations, which catalyze H_2 formation in the process of Fd_{red} oxidation, but do not allow the establishment of an ion gradient [7]. Furthermore, it is possible that part of the A_1 subcomplex of the ATP synthase was separated from the A_o subcomplex during the preparation of vesicles leading to proton flux without ATP synthesis. Hence, the *in vivo* quotient of ADP phosphorylation over H_2 formation is most probably much higher than the experimentally observed ATP/H_2 ratio.

Fd is an important cytoplasmic electron carrier in *Methanosarcina* species. The redox active protein is involved in the process of methanogenesis from H_2 + CO_2 (carboxymethanofuran reduction [31]), methylated compounds such as methanol and methylamines (oxidation of formylmethanofuran [32]), and from acetate (oxidation of CO bound to CODH/ACS [5]). The importance of ferredoxins in the metabolism is evident from the finding that the genome of *Ms. mazei* contains about 20 genes encoding these electron transport proteins [3]. Unfortunately, it is unknown which ferredoxin is the natural electron acceptor of the CODH/ACS. A couple of heterologously produced ferredoxins were tested for their ability to transfer electron from the CODH/ACS to the Ech hydrogenase but the electron transfer rates were low (not shown). Therefore, the ferredoxin from *Clostridium pasteurianum* was used in the experiments presented.

The free energy change associated with methane formation from one mol acetate is only -36 kJ/mol, which allows for the synthesis of less than 1 mol ATP. Thus, the loss of the Ech hydrogenase as a proton-translocating enzyme will have a dramatic effect on energy metabolism because these methanogens already live close to the thermodynamic limit. A severe impact can indeed be observed in *Methanosarcina* mutants lacking Ech hydrogenase. The *Ms. mazei* Δ*ech* mutant and the *Ms. barkeri* Δ*ech* mutant are unable to grow on acetate as sole energy source [20, 33]. Growth on trimethylamine as energy source is still possible for the *Ms. mazei* Δ*ech* mutant ($\Delta G^{o'}$= -76 kJ/mol CH_4), but with slower growth, less biomass and accelerated substrate consumption [20]. These results underline the importance of Fd_{red} oxidation by Ech hydrogenase in methanogenic pathways. In this context it is important to mention that *Ms. acetivorans*, a close relative of *Ms. mazei*, does not contain an Ech hydrogenase, but is able to grow on acetate. Since Fd_{red} is an essential intermediate in acetate metabolism, *Ms. acetivorans* must possess an alternative pathway for the utilization of this electron donor. It was suggested that in this organism the Rnf complex could substitute for the Ech hydrogenase [5].

By taking these data together, a new model of the ferredoxin:heterodisulfide oxidoreductase system can be devised (Fig. 4) and the long discussed hypothesis of ion translocation by Ech hydrogenase can be confirmed. The results presented here not only indicate that Ech hydrogenase acts as an additional energy coupling site in methanogenesis from acetate, but also identify the translocated ion as H^+. Both H^+ and Na^+ were feasible possibilities, but the results discussed above clearly exclude the involvement of Na^+ in energy conservation by Ech hydrogenase. Instead, the data strongly support the model of proton translocation by Ech hydrogenase, leading to a direct contribution to proton motive force. Thus, Ech hydrogenase acts as primary proton pump in Fd_{red}-dependent electron transport.

EXPERIMENTAL PROCEDURES

Preparation of inverted membrane vesicles, proteins and reagents

All experiments presented here were performed with *Ms. mazei* strain Gö1 (DSM 7222). Washed inverted membrane vesicles from *Ms. mazei* and *Ms. mazei* Δ*ech* [20] were prepared as described previously [7]. The strains were grown in 1 L glass bottles with 50 mM trimethylamine as substrate. The preparations were tested for the absence of enzyme activity with the cytoplasmic marker CODH/ACS to ensure the complete removal of cytoplasm from the membrane vesicles. Activity was tested by measuring the change of absorbance at 604 nm with 8.3 mM methylviologen, 5 % CO / 95 % N_2 in the gas phase and 300-500 µg vesicle preparation in 40 mM potassium phosphate buffer (including 5 mM dithioerythritol, 1 µg mL^{-1} resazurin, pH 7.0) in a total volume of 1 mL. Fd from *Clostridium pasteurianum* was isolated as described [34] with replacement of the last two steps (dialyzation, crystallization) by ultrafiltration. *Moorella thermoacetica* CODH/ACS was isolated as described [35] with the modifications specified in [20]. Synthesis of CoM-S-S-CoB was done according to [36].

Determination of ATP formation

ATP, ADP and AMP were supplied by Serva (Heidelberg, Germany). The inhibitors ETH157, DCCD and SF6847 and firefly lantern extract were supplied by Sigma-Aldrich (Schnelldorf, Germany). ETH157, DCCD and SF6847 were dissolved in 100% ethanol and used at final concentrations of 10-30 µM for ETH157 and SF6847, and 400 µM for DCCD.

To determine ATP formation, rubber stoppered glass vials were filled with 500 µL buffer A (20 mM potassium phosphate, 20 mM $MgSO_4$, 500 mM sucrose, 10 mM dithioerythritol, 1 µg mL^{-1} resazurin, pH 7.0), 5 % CO / 95 % N_2 in the 1.5 mL headspace, 500-700 µg washed inverted membrane vesicles, 33.5 µg ferredoxin, 150 nmol AMP, and 300 nmol ADP. Before starting the reaction by addition of 20 µg CODH/ACS, the reaction mixture was pre-incubated for 5 min at 37° C in a shaking water bath to inhibit the membrane-bound adenylate kinase. This enzyme catalyzes the formation of ATP and AMP from two ADP and can be fully inhibited by high concentrations of AMP [37] present in the reaction mixture. Upon start of the reaction, 10 µL samples were taken every 2.5 min. ATP detection was performed according to [38]. The samples were mixed with 700 µL 20 mM glycylglycine buffer, pH 8.0, containing 4 mM $MgSO_4$, and 100 µL firefly lantern extract. Emitted light was quantified after 10 s by a Luminescence Spectrometer LS50B (Perkin Elmer, USA) at 560 nm and the values compared to a standard curve.

Determination of H_2

For determining H_2 production rates, rubber stoppered glass vials were filled with 500 µL buffer A, 5 % CO / 95 % N_2 in the 1.5 mL headspace, 500-700 µg washed inverted membrane vesicles, 33.5 µg ferredoxin, 20 µg CODH/ACS, 150 nmol AMP, and 300 nmol ADP. At various reaction time points, 10 µL of the headspace were injected into a gas chromatograph (GC-14A, Shimadzu, Japan) with argon as carrier gas. Molecular hydrogen was analyzed by a thermal conductivity detector and quantified by comparison to a standard curve.

Acknowledgements

We thank Elisabeth Schwab for technical assistance and Paul Schweiger for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (grant De488/9-1).

REFERENCES

1. Khalil MAK & Rasmussen RA (1994) Global emissions of methane during the last several centuries. *Chemosphere* **29**, 833-842.
2. Reay DS (2003) Sinking methane. *Biologist (London)* **50**, 15-19.
3. Deppenmeier U, Johann A, Hartsch T, Merkl R, Schmitz RA, Martinez-Arias R, Henne A, Wiezer A, Bäumer S, Jacobi C, Brüggemann H, Lienard T, Christmann A, Bömeke M, Steckel S, Bhattacharyya A, Lykidis A, Overbeek R, Klenk HP, Gunsalus RP, Fritz HJ & Gottschalk G (2002) The genome of *Methanosarcina mazei*: evidence for lateral gene transfer between bacteria and archaea. *J Mol Microbiol Biotechnol* **4**, 453-461.
4. Deppenmeier U (2002) Redox-driven proton translocation in methanogenic Archaea. *Cell Mol Life Sci* **59**, 1513-1533.
5. Ferry JG & Lessner DJ (2008) Methanogenesis in marine sediments. *Ann N Y Acad Sci* **1125**, 147-157.
6. Meuer J, Bartoschek S, Koch J, Künkel A & Hedderich R (1999) Purification and catalytic properties of Ech hydrogenase from *Methanosarcina barkeri*. *Eur J Biochem* **265**, 325-335.
7. Ide T, Bäumer S & Deppenmeier U (1999) Energy conservation by the H_2:heterodisulfide

oxidoreductase from *Methanosarcina mazei* Gö1: identification of two proton-translocating segments. *J Bacteriol* **181**, 4076-4080.

8. Becher B, Müller V & Gottschalk G (1992) N5-methyl-tetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina* strain Gö1 is an Na(+)-translocating membrane protein. *J Bacteriol* **174**, 7656-7660.

9. Lienard T, Becher B, Marschall M, Bowien S & Gottschalk G (1996) Sodium ion translocation by N5-methyltetrahydromethanopterin: coenzyme M methyltransferase from *Methanosarcina mazei* Gö1 reconstituted in ether lipid liposomes. *Eur J Biochem* **239**, 857-864.

10. Hedderich R & Forzi L (2005) Energy-converting [NiFe] hydrogenases: more than just H_2 activation. *J Mol Microbiol Biotechnol* **10**, 92-104.

11. Bott M, Eikmanns B & Thauer R (1986) Coupling of carbon monoxide oxidation to CO_2 and H_2 with the phosphorylation of ADP in acetate-grown *Methanosarcina barkeri*. *Eur J Biochem* 159, 393-398.

12. Bott M & Thauer RK (1989) Proton translocation coupled to the oxidation of carbon monoxide to CO_2 and H_2 in *Methanosarcina barkeri*. *Eur J Biochem* **179**, 469-472.

13. Wilms R, Freiberg C, Wegerle E, Meier I, Mayer F & Müller V (1996) Subunit structure and organization of the genes of the A_1A_0 ATPase from the Archaeon *Methanosarcina mazei* Gö1. *J Biol Chem* **271**, 18843-18852.

14. Deppenmeier U, Blaut M, Mahlmann A & Gottschalk G (1990) Reduced coenzyme F_{420}: heterodisulfide oxidoreductase, a proton-translocating redox system in methanogenic bacteria. *Proc Natl Acad Sci U S A* **87**, 9449-9453.

15. Peinemann S, Blaut M & Gottschalk G (1989) ATP synthesis coupled to methane formation from methyl-CoM and H_2 catalyzed by vesicles of the methanogenic bacterial strain Gö1. *Eur J Biochem* **186**, 175-180.

16. Deppenmeier U, Blaut M, Schmidt B & Gottschalk G (1992) Purification and properties of a F_{420}-nonreactive, membrane-bound hydrogenase from *Methanosarcina* strain Gö1. *Arch Microbiol* **157**, 505-511.

17. Abken HJ, Tietze M, Brodersen J, Bäumer S, Beifuss U & Deppenmeier U (1998) Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Gö1. *J Bacteriol* **180**, 2027-2032.

18. Tersteegen A & Hedderich R (1999) *Methanobacterium thermoautotrophicum* encodes two multisubunit membrane-bound [NiFe] hydrogenases. Transcription of the operons and sequence analysis of the deduced proteins. *Eur J Biochem* **264**, 930-943.

19. Stojanowic A & Hedderich R (2004) CO_2 reduction to the level of formylmethanofuran in *Methanosarcina barkeri* is non-energy driven when CO is the electron donor. *FEMS Microbiol Lett* **235**, 163-167.

20. Welte C, Kallnik V, Grapp M, Bender G, Ragsdale S & Deppenmeier U (2010) Function of Ech hydrogenase in ferredoxin-dependent, membrane-bound electron transport in *Methanosarcina mazei*. *J Bacteriol* **192**, 674-678.

21. Sapra R, Bagramyan K & Adams MW (2003) A simple energy-conserving system: proton reduction coupled to proton translocation. *Proc Natl Acad Sci U S A* **100**, 7545-7550.

22. Fox JD, Kerby RL, Roberts GP & Ludden PW (1996) Characterization of the CO-induced, CO-tolerant hydrogenase from *Rhodospirillum rubrum* and the gene encoding the large subunit of the enzyme. *J Bacteriol* **178**, 1515-1524.

23. Soboh B, Linder D & Hedderich R (2002) Purification and catalytic properties of a CO-oxidizing:H_2-evolving enzyme complex from *Carboxydothermus hydrogenoformans*. *Eur J Biochem* **269**, 5712-5721.

24. Böhm R, Sauter M & Böck A (1990) Nucleotide sequence and expression of an operon in *Escherichia coli* coding for formate hydrogenlyase components. *Mol Microbiol* **4**, 231-243.

25. Sauter M, Böhm R & Böck A (1992) Mutational analysis of the operon (hyc) determining hydrogenase 3 formation in *Escherichia coli*. *Mol Microbiol* **6**, 1523-1532.

26. Andrews SC, Berks BC, McClay J, Ambler A, Quail MA, Golby P & Guest JR (1997) A 12-cistron *Escherichia coli* operon (hyf) encoding a putative proton-translocating formate hydrogenlyase system. *Microbiology* **143** (Pt 11), 3633-3647.

27. Pisa KY, Weidner C, Maischak H, Kavermann H & Müller V (2007) The coupling ion of the methanoarchaeal ATP synthases: H^+ vs. Na^+ in the A_1A_o synthase from the archaeon *Methanosarcina mazei* Gö1. *FEMS Microbiol Lett* 277, 56-63.

28. Pisa KY, Huber H, Thomm M & Müller V (2007) A sodium ion-dependent A_1A_o ATP synthase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *FEBS J* 274, 3928-3938.

29. Peinemann S (1989) Kopplung von ATP-Synthese und Methanogenese in Vesikelpräparationen des methanogenen Bakteriums Stamm Gö1. PhD thesis, University of Göttingen, Germany.

30. Cross RL & Müller V (2004) The evolution of A-, F-, and V-type ATP synthases and ATPases:

reversals in function and changes in the H^+/ATP coupling ratio. *FEBS Lett* 576, 1-4.

31. Bartoschek S, Vorholt JA, Thauer RK, Geierstanger BH & Griesinger C (2000) N-carboxymethanofuran (carbamate) formation from methanofuran and CO_2 in methanogenic archaea. Thermodynamics and kinetics of the spontaneous reaction. *Eur J Biochem* **267**, 3130-3138.

32. Deppenmeier U (2002) The unique biochemistry of methanogenesis. *Prog Nucleic Acid Res Mol Biol* **71**, 223-283.

33. Meuer J, Kuettner HC, Zhang JK, Hedderich R & Metcalf WW (2002) Genetic analysis of the archaeon Methanosarcina barkeri Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. *Proc Natl Acad Sci U S A* **99**, 5632-5637.

34. Mortenson LE (1964) Purification and analysis of ferredoxin from *Clostridium pasteurianum*. *Biochimica et Biophysica Acta (BBA) - Specialized Section on Enzymological Subjects* **81**, 71-77.

35. Ragsdale SW, Ljungdahl LG & Der Vartanian DV (1983) Isolation of carbon monoxide dehydrogenase from *Acetobacterium woodii* and comparison of its properties with those of the *Clostridium thermoaceticum* enzyme. *J Bacteriol* **155**, 1224-1237.

36. Ellermann J, Hedderich R, Bocher R & Thauer RK (1988) The final step in methane formation. Investigations with highly purified methyl-CoM reductase (component C) from *Methanobacterium thermoautotrophicum* (strain Marburg). *Eur J Biochem* **172**, 669-677.

37. Bomsel JL & Pradet A (1968) Study of adenosine 5'-mono-, di- and triphosphates in plant tissues. IV. Regulation of the level of nucleotides, in vivo, by adenylate kinase: Theoretical and experimental study. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **162**, 230-242.

38. Kimmich GA, Randles J & Brand JS (1975) Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. *Anal Biochem* **69**, 187-206.

Chapter 4

Re-evaluation of the function of the F_{420} dehydrogenase in electron transport of *Methanosarcina mazei*

The ferredoxin: heterodisulfide oxidoreductase system of *Ms. mazei* and *Ms. barkeri* makes use of a hydrogen cycling mechanism via Ech hydrogenase and F_{420} non-reducing hydrogenase. The membrane-bound $F_{420}H_2$ dehydrogenase was found to be responsible for the oxidation of $F_{420}H_2$ that is produced during methylotrophic methanogenesis and is well studied in *Ms. mazei* (Bäumer *et al.*, 2000, Brodersen *et al.*, 1999). Conversely, it was recently suggested that in *Ms. barkeri* the cytoplasmic F_{420} hydrogenase, not the $F_{420}H_2$ dehydrogenase, is responsible for $F_{420}H_2$ oxidation with concomitant H_2 production. This system would also make use of a hydrogen cycling mechanism as shown for the ferredoxin: heterodisulfide oxidoreductase (Kulkarni *et al.*, 2009). In this publication, the role of the second hydrogen cycling mechanism in comparison to the $F_{420}H_2$ dehydrogenase (Fpo) reaction was investigated. With the help of two *Ms. mazei fpo* knockout mutants (Δ*fpoA-O*, Δ*fpoF*) it was found that *Ms. mazei* and *Ms. barkeri* differ in their preference for hydrogen cycling from $F_{420}H_2$, and that the majority of *Methanosarcina* sp. probably do not depend on hydrogen as obligate intermediate in $F_{420}H_2$ oxidation. The second part of the publication describes the activity of the soluble F-subunit of Fpo. It catalyzes the redox reaction of ferredoxin with F_{420}. A protein catalyzing this reaction had been hypothesized as being responsible for the survival of the *Ms. mazei* and *Ms. barkeri* Δ*ech* mutants because the accumulation of reduced ferredoxin had to be prevented in these strains. Furthermore, the interconversion of different reducing equivalents could be beneficial and allow more flexibility for the switch of growth substrates.

Welte and Deppenmeier (2011) *FEBS J* **278**:1277-1287

Re-evaluation of the function of the F_{420} dehydrogenase in electron transport of *Methanosarcina mazei*

Cornelia Welte and Uwe Deppenmeier

Institute of Microbiology and Biotechnology, University of Bonn, Meckenheimer Allee 168, 53115 Bonn, Germany

***Methanosarcina mazei* is a methanogenic archaeon that is able to thrive on various substrates and thus contains a variety of redox active proteins involved in both cytoplasmic and membrane-bound electron transport. The organism possesses a complex branched respiratory chain that has the ability to utilize different electron donors. In this study, two knockout mutants of the membrane-bound F_{420} dehydrogenase (ΔfpoF and ΔfpoA-O) were constructed and analyzed. They exhibited severe growth deficiencies with trimethylamine, but not with acetate as substrate. In cell lysates of the *fpo* mutants, the F_{420}: heterodisulfide oxidoreductase activity was strongly reduced although soluble F_{420} hydrogenase was still present. This led to the conclusion that the predominant part of cellular $F_{420}H_2$ oxidation in *Ms. mazei* is performed by F_{420} dehydrogenase. Enzyme assays of cytoplasmic fractions revealed that ferredoxin: F_{420} oxidoreductase activity was essentially absent in the ΔfpoF mutant. Subsequently, FpoF was produced in *Escherichia coli* and purified for further characterization. The purified FpoF protein catalyzed the ferredoxin: F_{420} oxidoreductase reaction with high specificity (K_m for reduced ferredoxin 0.5 µM) but low velocity (v_{max} 225 mU mg^{-1}) and was present in the *Ms. mazei* cytoplasm in considerable amounts. Consequently, soluble FpoF might participate in electron carrier equilibrium and facilitate survival of the *Ms. mazei* Δech mutant that lacks the membrane-bound ferredoxin-oxidizing Ech hydrogenase.**

INTRODUCTION

Methanogenic archaea are one of the key groups in the global carbon cycle because they metabolize the final products of the anaerobic food chain (H_2, CO_2 and acetate) using unusual enzymes and cofactors in the so-called methanogenic pathway. The final product of all methanogenic pathways is methane and every year millions of tons of this highly potent greenhouse gas reach the atmosphere and contribute to global warming. Apart from their vast distribution in nature, methanogens are responsible for the production of CH_4 in anaerobic digesters of biogas plants. The process of biomethanation is a viable alternative to fossil fuels and has great potential as an important renewable energy source.

In principle, three different growth strategies in methanogenesis have evolved: hydrogenotrophic, methylotrophic and aceticlastic methanogenesis, using H_2 + CO_2, methylated compounds and acetate as substrates, respectively. The core of all methanogenic pathways is very similar, but there are differences in the source of reducing equivalents and the mode of how electrons are channelled into the methanogenic respiratory chain. *Methanosarcina mazei* Gö1 (*Ms. mazei*) is a model organism for methanogenisis because it can grow hydrogenotrophically, methylotrophically, and aceticlastically. Its respiratory chain comprises three energy-conserving oxidoreductase systems that lead to the formation of an electrochemical proton gradient and ATP synthesis by the A_1A_o ATP synthase [1]. The energy transducing systems are referred to as $F_{420}H_2$: heterodisulfide oxidoreductase, H_2: heterodisulfide oxidoreductase and ferredoxin: heterodisulfide oxidoreductase, mirroring the three possible electron input compounds ($F_{420}H_2$, H_2, reduced ferredoxin (Fd_{red})). Ferredoxin (Fd) is reduced during methylotrophic and aceticlastic methanogenesis, and is oxidized by Ech hydrogenase as part of the ferredoxin: heterodisulfide oxidoreductase system. $F_{420}H_2$ (the reduced form of a 8-hydroxy-5-deazaflavin) is formed in the course of methyl group oxidation in the methylotrophic pathway, or in the cytoplasm of hydrogenotrophically growing methanogens by F_{420} hydrogenase (Frh). This

Tab. 1: Methane formation by resting cell suspensions of *Ms. mazei.*

washed cells	substrate	methane formation (nmol min^{-1} mg $protein^{-1}$)
wildtype	trimethylamine	196 ± 8
ΔfpoF	trimethylamine	107 ± 11
ΔfpoA-O	trimethylamine	105 ±19
wildtype	trimethylamine + H_2	185 ± 10
ΔfpoF	trimethylamine + H_2	190 ± 17
ΔfpoA-O	trimethylamine + H_2	186 ± 2

enzyme oxidizes H_2 with concomitant F_{420} reduction providing $F_{420}H_2$ for CO_2 reduction.

The $F_{420}H_2$: heterodisulfide oxidoreductase used under methyltrophic growth conditions consists of the $F_{420}H_2$ dehydrogenase (Fpo) and the heterodisulfide reductase [2-3]. Here we describe the characteristics of two Fpo mutants (ΔfpoF and ΔfpoA-O) and the effects of these deletions on the process of energy conservation. Furthermore, it is shown that the protein FpoF functions as input module of the Fpo complex. In a soluble form it is able to catalyze the reduction of coenzyme F_{420} with reduced ferredoxin as electron donor, providing a direct link between the redox carriers of hydrogenotrophic and aceticlastic methanogenesis.

RESULTS

Electron transport in Δfpo mutants

The $F_{420}H_2$ dehydrogenase (Fpo) is predicted to be a key enzyme in methylotrophic methanogenesis of *Ms. mazei* that catalyses the membrane-bound oxidation of $F_{420}H_2$ and the reduction of methanophenazine as membrane-soluble redox carrier with concomitant extrusion of 2 H^+/1 e^- [2-3]. However, Kulkarni *et al.* [4] presented evidence that in *Ms. barkeri*, a close relative of *Ms. mazei*, the cytoplasmic F_{420} hydrogenase (Frh) is to a high degree responsible for $F_{420}H_2$ oxidation, thereby producing molecular hydrogen, which can be oxidized by the membrane-bound F_{420}-nonreducing hydrogenase (Vho). Hence, both Fpo and Frh (in combination with Vho) may function as electron input modules channelling electrons into the respiratory chain.

To evaluate electron transport from $F_{420}H_2$ in *Ms. mazei* in more detail, two mutants with deletion of genes encoding the $F_{420}H_2$ dehydrogenase ΔfpoF (= Δ0627) and ΔfpoA-O (= Δ2491-2479) were constructed. Slower growth rates were observed for the ΔfpoF and the ΔfpoA-O mutants (doubling times 11.3 h and 11.1h) in comparison to the wildtype (doubling time 7.7 h) with trimethylamine as substrate. The final optical densities of the cultures were almost identical. In summary, the electron transport deficiency of the Δfpo mutants was reflected in their growth abilities as they grew slower but generated the same amount of biomass from trimethylamine compared to the parental strain. As expected, both mutants grew on acetate or trimethylamine plus H_2 with a growth rate and yield similar to the parental strain indicating that Fpo does play a major role in the oxidative part of methylotrophic methanogenesis.

The effect of the mutations was also analyzed using resting cell suspensions of *Ms. mazei* (Tab. 1). Methane formation from trimethylamine was measured under an atmosphere of nitrogen and it became evident that the ΔfpoF and the ΔfpoA-O mutants revealed only half of the activity compared to the parental strain. In contrast, no differences in methane production were observed when trimethylamine was converted to methane in the presence of molecular hydrogen.

To evaluate the reason for the different growth rates and methane formation rates with trimethylamine as substrate, cell lysates and washed membrane preparations of the wildtype and the mutant strains were prepared and the coupled redox reaction of $F_{420}H_2$ oxidation with heterodisulfide reduction ($F_{420}H_2$: heterodisulfide oxidoreductase) was assayed (eq. 1).

Eq. 1: $F_{420}H_2$ + CoM-S-S-CoB → F_{420} + HS-CoM + HS-CoB

$F_{420}H_2$: heterodisulfide oxidoreductase activity was exclusively found in the membrane fraction of the wildtype with a specific activity of about 130 mU mg^{-1} membrane protein, and was < 1 mU mg^{-1} membrane protein in the membrane fractions of the ΔfpoF and ΔfpoA-O mutant

Tab 2: Activities of the F_{420} hydrogenase in cell lysates. Tests were conducted as specified in the "Materials and Methods" part with 10 µM $F_{420}H_2$ or 100 % H_2 in the head space as electron donors, and 10^{-7} M H^+ (pH 7) or 10 µM F_{420} as electron acceptors.

Cell lysate	electron donor	electron acceptor	reduction of electron acceptor (nmol min^{-1} mg^{-1})
Ms. mazei [1]	$F_{420}H_2$	H^+	0.65
Ms. mazei ΔfpoA-O	$F_{420}H_2$	H^+	0.55
Ms. mazei ΔfpoF	$F_{420}H_2$	H^+	0.45
Ms. mazei [1]	H_2	F_{420}	35
Ms. barkeri[1]	H_2	F_{420}	360

1) wildtype

strains (not shown). The results are in agreement with the hypothesis that Fpo is the only membrane-integral enzyme that is able to channel electron from $F_{420}H_2$ directly into the respiratory chain [1]. However, $F_{420}H_2$ is a stringent intermediate in methanogenesis from methylated substrates, such as trimethylamine, where part of the methyl groups are oxidized to CO_2 and electrons are transferred to F_{420} in the course of the methylene-H_4SPT reductase (eq. 2) and dehydrogenase reaction (eq. 3) [5-6].

Eq. 2: methyl-H_4SPT + F_{420} → methylene-H_4SPT + $F_{420}H_2$
Eq. 3: methylene-H_4SPT + F_{420} → methenyl-H_4SPT + $F_{420}H_2$

Accordingly, $F_{420}H_2$ has to be re-oxidized in the wildtype and the mutant strains. Since there is no membrane-bound $F_{420}H_2$ dehydrogenase activity, the two Δfpo mutant strains obviously depend on alternative components that catalyse this reaction. Indeed, the cell lysate of the two mutant strains exhibited $F_{420}H_2$-dependent CoM-S-S-CoB reductase activity of approximately 4-5 mU mg^{-1} protein (Fig. 1), whereas in cell lysate of the parental strain the activity was approximately 50 mU mg^{-1} protein. These findings clearly indicate that the predominant part of $F_{420}H_2$ oxidation is performed by the membrane-bound Fpo complex (approximately 90%) and only a minor part (approximately 10%) of $F_{420}H_2$ oxidation is accomplished by alternative enzymes.

Kulkani *et al.* [4] showed that in *Ms. barkeri*, the soluble F_{420} hydrogenase (Frh) is the key enzyme in reoxidation of $F_{420}H_2$.

Eq. 4: $F_{420}H_2 \rightarrow F_{420} + H_2$ $\Delta G_0' = +11.6$ kJ / mol
Eq. 5: H_2 + CoM-S-S-CoB → HS-CoM + HS-CoB $\Delta G_0' = -49.2$ kJ / mol

This finding raised the question whether Frh is also involved in cofactor regeneration in *Ms. mazei*. As evident form Tab. 2 electrons from $F_{420}H_2$ were transferred to H^+ in the cell lysates and escape as H_2 when no external electron acceptor (heterodisulfide) was added to the cell extracts. The activities were rather low (about 0.5 mU mg^{-1} cellular protein) but were approximately the same in the parental strain and the deletion mutants. In contrast, the reverse reaction with H_2 as electron donor was catalyzed by Frh with 35 mU mg^{-1} cellular protein and therefore was more than 50-fold faster (Tab. 2). Nevertheless, the Fpo mutants are obviously able to take advantage of the H_2 evolving activity of the F_{420} hydrogenase when $F_{420}H_2$ accumulates in these mutants. However, it is evident that the low hydrogen producing activity of the F_{420} hydrogenase cannot compensate the activity of the $F_{420}H_2$ dehydrogenase and leads to impaired growth of the ΔfpoF and ΔfpoA-O mutants. This situation might be different in *Ms. barkeri* because the cell extract of this organism had a F_{420} hydrogenase activity of about 360 mU mg^{-1} protein and was therefore 10-fold higher compared to the activity found in cell extracts of *Ms. mazei* (Tab. 2). This finding is in line with the observation that hydrogen is a preferred intermediate in the energy-conserving electron transport chain of *Ms. barkeri* [4].

A second possibility of $F_{420}H_2$ oxidation in the fpo mutants is the utilization of NAD(P) as electron acceptor as catalysed by a F_{420}: NAD(P) oxidoreductase, which was characterized from *Methanococcus vannielii* [7], *Archaeoglobus fulgidus* [8], *Methanobacterium thermoautotrophicum* [9] and *Methanosphaera stadtmanae* [10]. *Ms. mazei* is also able to produce such an enzyme, which is encoded by the gene *mm0977* (unpublished results). In the course of the reaction reduced nicotinamide adenine dinucleotide would be formed. However, neither NADPH nor NADH function as electron donors for the membrane-bound electron

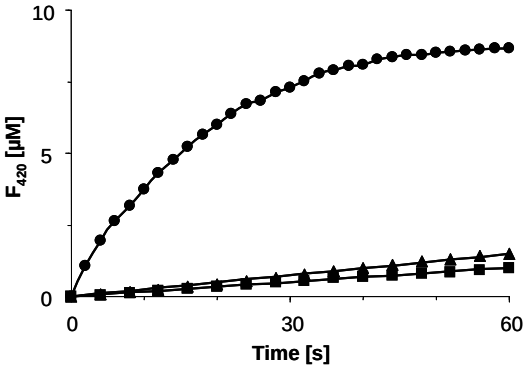

Figure 1: $F_{420}H_2$: heterodisulfide oxidoreductase activity in *Ms. mazei* cell lysate. Cuvettes contained 600 µL buffer A, 10 µM $F_{420}H_2$, 250 µg cell lysate, 33 µM heterodisulfide under a N_2 atmosphere. ●, wildtype cell lysate; ▲, ΔfpoA-O cell lysate; ■, ΔfpoF cell lysate.

transport systems in *Ms. mazei*. Therefore, the F_{420}: NAD(P) oxidoreductase cannot participate in energy metabolism. In summary, there is a wealth of evidence that the $F_{420}H_2$ dehydrogenase is of major importance for $F_{420}H_2$-dependent heterodisulfide reduction in *Ms. mazei*. The possible electron transfer via H_2 in the ΔfpoF and ΔfpoA-O mutants is most likely only a rescue pathway that allows relatively slow growth of the cells with increased doubling times.

Electron transport in Δech mutants

Another question in methanogenic bioenergetics concerns the fate of Fd_{red} that is produced in aceticlastic and methylotrophic methanogenesis in the course of the acetyl-CoA synthase/CO dehydrogenase and formylmethanofuran dehydrogenase reaction, respectively.

Eq. 6: Acetyl-CoA + H_4SPT + 2 Fd → CH_3-H_4SPT + CO_2 + 2 Fd_{red} + HS-CoA
Eq. 7: Formyl-MF + 2 Fd → MF + CO_2 + 2 Fd_{red}

In many *Methanosarcina* sp. Fd_{red} is oxidized by the membrane-bound, proton-translocating and H_2-forming Ech hydrogenase [11]. The produced H_2 is scavenged by the membrane-bound hydrogenase (Vho/Vht). Surprisingly, both *Ms. mazei* and *Ms. barkeri* Δech mutants are still capable of growth on methylated amines, where Fd_{red} provides one third of the reducing equivalents needed for heterodisulfide reduction. Although Ech hydrogenase is evidently missing, Fd_{red} can still be oxidized by an unknown mechanism. To shed light on this phenomenon, the *Ms. mazei* mutants and wildtype were analyzed for enzymatic activities producing reduced F_{420} from Fd_{red}.

Eq. 8: 2 Fd_{red} + F_{420} + 2 H^+ → 2 Fd + $F_{420}H_2$ ΔG_0' = -11.6 kJ/mol

Membrane fractions of the wildtype and all deletion mutants did not catalyse the Fd: F_{420} oxidoreductase reaction. In contrast, direct measurement of Fd: F_{420} oxidoreductase activity in cytoplasmic fractions revealed that Fd_{red}-dependent F_{420} reduction occurred in the wildtype, Δech mutant and the ΔfpoA-O mutant (Fig. 2). Interestingly this activity was essentially absent in the cytoplasmic fraction of the ΔfpoF mutant.

This observation prompted investigation into the function of FpoF in more detail. The corresponding gene, *mm0627,* was expressed in *E. coli* and the protein was purified by affinity chromatography to apparent homogeneity (Fig. S1). UV-Vis spectra revealed the presence of flavins and iron-sulfur clusters (Fig. S2). The determination of iron and sulfur yielded 8.7 ± 0.1 mol non-heme iron and 5.1 ± 0.2 mol acid-labile sulfur per mol protein. These findings indicate the presence of two [4Fe-4S]-clusters predicted from the amino acid sequence and are in accordance with the FeS cluster content of the homologous protein FqoF from *Archaeoglobus fulgidus* [12]. Finally, HPLC analysis revealed the presence of 1.0 ± 0.1 mol FAD per mol protein. The purified FpoF protein from *Ms. mazei* was able to oxidize clostridial Fd_{red} and reduce F_{420}. The kinetic parameters of the reaction exhibit a maximal velocity (V_{max}) of 225 mU mg^{-1} protein and a K_M of 2 µM and 0.5 µM for F_{420} and Fd_{red}, respectively (Fig. 3). The relatively low activity of purified FpoF is probably based on the fact that a clostridial ferredoxin was used as electron donor, which might be responsible for an impaired electron transfer onto FpoF. Further studies will indicate which of the *Methanosarcina*-ferredoxins functions as natural electron carrier.

Localization and dual function of FpoF

As a prerequisite for the *in vivo* action of FpoF as Fd: F_{420} oxidoreductase, the protein has to be present in the cytoplasm in addition to its presence at the membrane-bound Fpo complex. To examine this, antibodies directed against FpoF were produced using purified FpoF as antigen, and used in subsequent immunoblotting experiments. Membrane-free cytoplasm and washed membrane fractions were applied to SDS-PAGE and immunoblotting. Known concentrations of FpoF were also applied and were used to quantify FpoF based on relative band intensities (Fig. 4). When the bands for the cytoplasm and the membrane fraction were

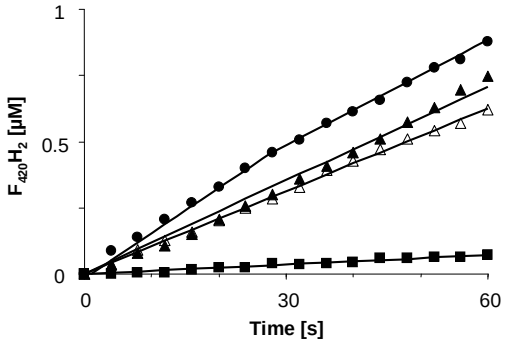

Figure 2: Fd: F_{420} oxidoreductase activity in *Ms. mazei* cytoplasm. Assays contained 500 µg cytoplasmic protein, 15 µM F_{420}, 50 µg CO dehydrogenase, 5 µg clostridial Fd in 600 µL buffer A under a 5 % CO / 95 % N_2 atmosphere. ●, wildtype cytoplasm; ▲, ΔfpoA-O cytoplasm; Δ, Δech cytoplasm; ■, ΔfpoF cytoplasm.

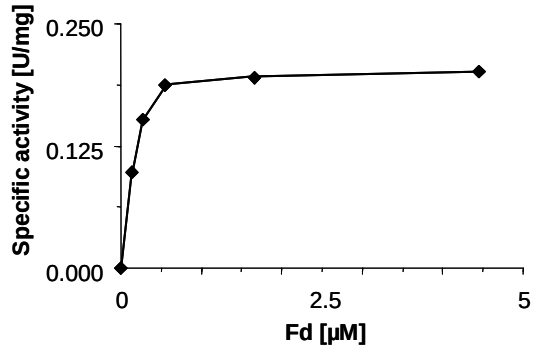

Figure 3: Kinetic parameters of the purified Fd: F_{420} oxidoreductase FpoF. Dependence of the activity on the Fd concentration. Assays contained 50 µg CO dehydrogenase and 10 µM F_{420} in 600 µL buffer A under a 5 % CO / 95 % N_2 atmosphere, as well as variable amounts of Fd.

compared to the calibration curve, about 76 ng per µl membrane preparation could be identified as FpoF and about 4 ng FpoF per µl of cytoplasm could be detected. Taking into account the protein concentrations of these preparations with respect to the total cellular protein content, theamount of soluble FpoF was about 0.9 µg/mg protein and 3 µg/mg protein were present in the membrane fraction. The cytoplasmic fraction was also tested for contaminations with membrane proteins by analysing the reduction of CoM-S-S-CoB as catalysed by the membrane-bound heterodisulfide reductase (HdrDE) to exclude the possibility that FpoF connected to the Fpo complex interfered with the quantification of soluble FpoF. The specific activity of HdrDE was < 1 mU/mg protein indicating that the cytoplasmic fraction was free of membrane particles. Hence, it is obvious that the major part of the total FpoF

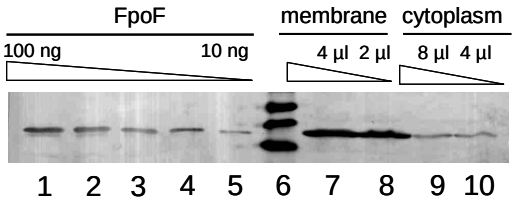

Figure 4: Immunoblot used for FpoF quantification. Proteins blotted onto nitrocellulose membrane were detected with rabbit-anti-FpoF and HRP-conjugated goat-anti-rabbit IgG. Lanes 1-5: 10 – 100 ng FpoF, lane 6: molecular mass standard; lane 7-8: solubilized membrane preparation from *Ms. mazei*, lane 9-10: membrane-free cytoplasm from *Ms. mazei*.

protein was membrane-bound (about 75%) but a significant amount of this protein was also present in the cytoplasmic fraction (about 25%).

These findings led to the hypothesis that FpoF might have dual function. First, when it is connected to the membrane integral Fpo complex, it functions as electron input module of the $F_{420}H_2$ dehydrogenase. Second as a soluble single subunit it is involved in the process of re-oxidation of Fd_{red} in the cytoplasm whereby producing reduced F_{420} that is then re-oxidized by the complete Fpo complex. In summary, these experiments led to the conclusion that soluble FpoF can function as Fd: F_{420} oxidoreductase when Fd_{red} accumulates in the cytoplasm as predicted for the Δech mutant.

DISCUSSION

Reduced coenzyme F_{420} as electron donor of the respiratory chain

$F_{420}H_2$ is the key electron donor in methanogens and is formed by the reduction of F_{420} in methylotrophic and hydrogenotrophic methanogenesis. When *Ms. mazei* grows on H_2/CO_2, H_2 can take two routes into the metabolism: it is either oxidized by a membrane-bound, proton-translocating H_2: heterodisulfide oxidoreductase system (Fig. 5a), or it is oxidized by a soluble, non-respiratory F_{420} hydrogenase (Frh) that reduces F_{420} [13-18]. The resulting $F_{420}H_2$ is mainly used for the reduction of CO_2 in the methanogenic pathway where the heterodisulfide CoM-S-S-CoB as terminal electron acceptor is formed, which is reduced by the energy conserving H_2: heterodisulfide oxidoreductase. In methylotrophic methanogenesis $F_{420}H_2$ is formed during methyl

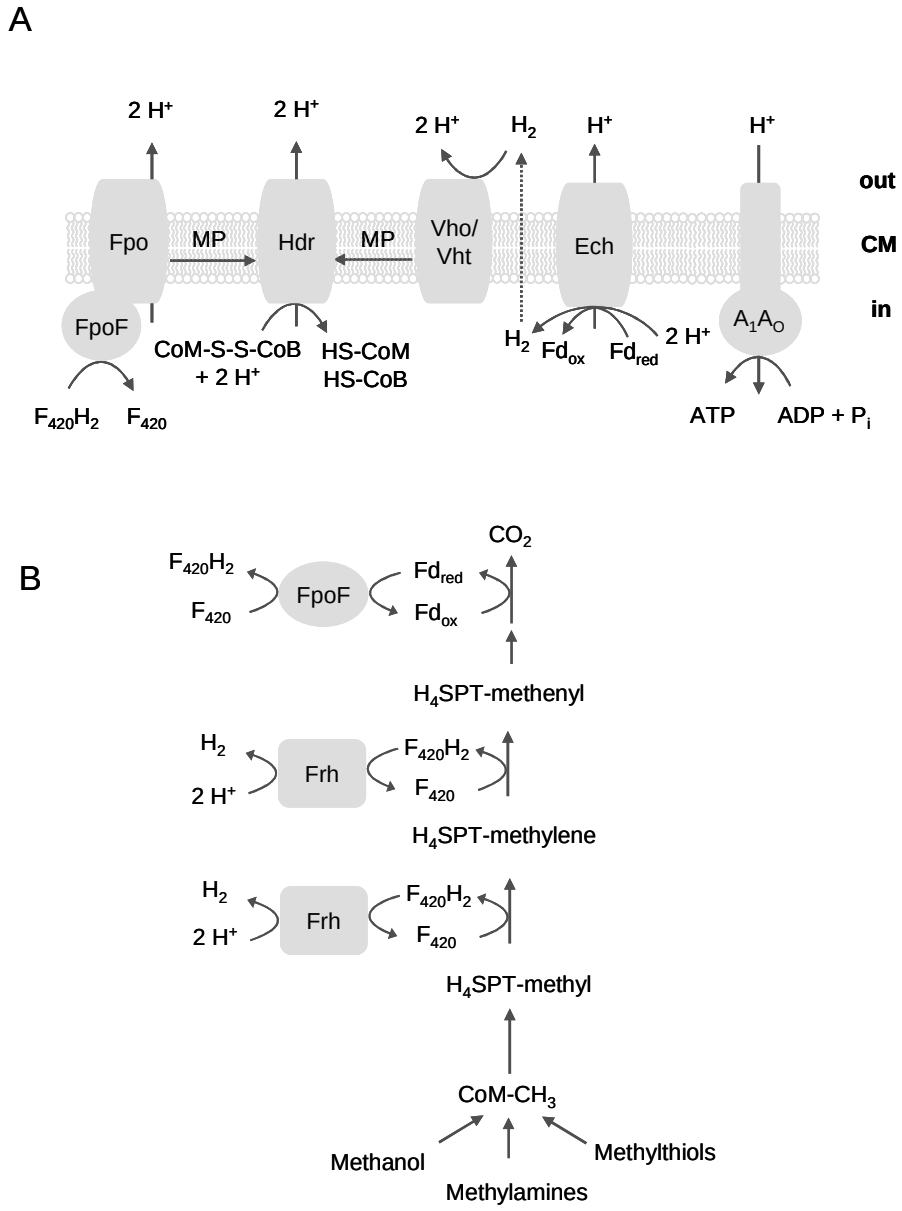

Figure 5: Model of the branched electron transport pathway in *Methanosarcina mazei*. (A) membrane-bound and (B) cytoplasmic electron transport in *Ms. mazei*. Vho/Vht, F_{420} non-reducing hydrogenase; Hdr, heterodisulfide reductase; Frh, F_{420} (reducing) hydrogenase; Fpo, $F_{420}H_2$ dehydrogenase; FpoF, F-subunit of Fpo; MP, methanophenazine; CoM, Coenzyme M; H_4SPT, tetrahydrosarcinapterin; A_1A_O, A_1A_O ATP synthase.

group oxidation to CO_2 (Fig. 5b). In principle, the CO_2 reduction pathway of the hydrogenotrophic methanogens acts in reverse, and $F_{420}H_2$ can be used in the respiratory chain (Fig. 5b). In *Ms. mazei*, the $F_{420}H_2$ dehydrogenase (Fpo) was characterized in detail as part of the membrane-bound energy-conserving $F_{420}H_2$: heterodisulfide oxidoreductase system [2-3, 14]. The soluble F_{420} hydrogenase (Frh) is also present in this organism under methylotrophic growth conditions [19]. The question arose whether this enzyme can act in the reverse direction as compared to hydrogenotrophic methanogenesis, namely producing H_2 through the oxidation of $F_{420}H_2$ (Fig. 5b). As shown before, the Frh activity in cells extracts of the parental strain and the mutants was low probably because the overall reaction is thermodynamically unfavourable under standard conditions. The activity might be higher when the reaction is coupled to the exergonic reduction of the heterodisulfide (ΔG_0' = -42.1 kJ/mol). Evidence in favour of this hypothesis came from the finding that the rate of $F_{420}H_2$ dependent heterodisulfide reduction in cell lysates of the ΔfpoA-O or ΔfpoF mutant was in the range of 5 mU mg^{-1} protein and hence ten-fold higher than the rate of H_2 production from $F_{420}H_2$ in the absence of CoM-S-S-CoB. The produced H_2

could then be used by the membrane-bound H_2: heterodisulfide oxidoreductase and would yield the same amount of translocated protons as compared to the $F_{420}H_2$: heterodisulfide oxidoreductase system (Fig. 5a).

In contrast to the *fpo* mutants, the wildtype exhibited an activity of about 50 mU mg^{-1} protein with the substrate combination $F_{420}H_2$ plus heterodisulfide. This clearly demonstrates that, under methylotrophic growth conditions, 90 % of the cellular $F_{420}H_2$ oxidation in *Ms. mazei* is performed by the $F_{420}H_2$ dehydrogenase connected to heterodisulfide reductase via methanophenazine. Only 10 % of the cellular $F_{420}H_2$ oxidation is accomplished by the cytoplasmic F_{420} hydrogenase, leading to H_2 efflux that is scavenged by the membrane-bound H_2: heterodisulfide oxidoreductase (Fig. 5 a/b).

As already indicated the $F_{420}H_2$ oxidizing/H_2 evolving activity of cell lysates was only about 0.5 mU mg^{-1} cellular protein. However, the reverse reaction (i.e. oxidation of H_2 coupled to the reduction of F_{420}) is catalyzed at a much higher activity of about 35 mU mg^{-1} cellular protein and is probably physiological when hydrogenotrophic methanogenesis is performed. In *Ms. barkeri*, H_2: F_{420} oxidoreductase activity was observed at a much higher activity in cell lysates. We found an activity of about 360 mU mg^{-1} protein and Michel *et al.* [20] even reported 870 mU mg^{-1} protein in methanol-grown cells. This is a 10-25-fold higher activity than observed in *Ms. mazei*, and an indicator for a stronger activity of the reverse reaction, namely $F_{420}H_2$ oxidation with H_2 production. It is tempting to speculate that the role of Frh in *Ms. barkeri* under methylotrophic growth conditions is greater than the role for the same enzyme in *Ms. mazei*. In fact this was actually observed by Kulkarni *et al.* [4] who reported that in *Ms. barkeri* knockouts of *fpoA-O* or *fpoF* do not significantly alter growth parameters, whereas the same knockouts in *Ms. mazei* strongly decreased the doubling time. In *Methanosarcina acetivorans*, a close relative to both *Ms. mazei* and *Ms. barkeri*, the apparent lack of membrane-bound hydrogenase activity [21-23] makes the route of $F_{420}H_2$ oxidation by Frh impossible. In addition, only very low hydrogenase activities were observed in *Ms. thermophila* [24] and obligate methylotrophic methanogens such as *Methanolobus tindarius* [25] and *Methanococcoides burtonii* [26-27]. Taking these findings together, we conclude that almost all methylotrophic methanogens rely on the $F_{420}H_2$ dehydrogenase to feed reducing equivalent into the respiratory chain (Fig. 5a). Obviously, the only known exception is *Ms. barkeri* where reducing equivalents from methyl group oxidation seem to be preferentially passed to molecular H_2 by the cytoplasmic F_{420}-reducing hydrogenase. Hence, *Ms. barkeri* cannot be used as a model for the principal electron transport chain of methylotrophic methanogens and a re-analysis of energy-conservation mechanisms in *Methanosarcina* species as suggested by Kulkarni et al. [4] is not warranted.

Dual function of subunit FpoF of the Fpo complex

The cluster encoding the $F_{420}H_2$ dehydrogenase of *Ms. mazei* and related *Methanosarcina* strains is composed of 12 genes (*fpoA-O*) [3]. Analysis of the Fpo subunits showed that the enzyme is highly similar to proton-translocating NADH dehydrogenases (NDH-1). The gene products FpoAHJKLMN are hydrophobic and are homologous to subunits that form the membrane integral module of NDH-1. FpoBCDI have their counterparts in the amphipatic membrane-associated module of NDH-1. Homologues to the hydrophilic NADH-oxidizing subunit of NDH-1 are not present in *Ms. mazei*. Instead, the gene product FpoF is responsible for $F_{420}H_2$ oxidation and functions as the electron input device [3]. Interestingly, the gene *fpoF* is not part of the operon and is located at a different site on the chromosome.

Previously it was only known that the complex of FpoA-O and FpoF has a role in $F_{420}H_2$ oxidation, but is not involved in the reaction with Fd. This fact still holds true for the entire complex, but the single subunit FpoF may also interact with Fd_{red} (Fig 5b). A slow reduction of F_{420} with Fd_{red} as substrate could be detected in the cytoplasm of the parental strain and the ΔfpoA-O and Δech mutants. In the ΔfpoF mutant this activity was basically absent. Finally we showed that the purified FpoF functions as Fd: F_{420} oxidoreductase. The results of the immunoblotting experiments indicated that FpoF is present in the cytoplasm in relatively high concentrations. This fact cannot be explained by trafficking of premature FpoF intended for binding to the FpoA-O complex but points to a second function of the soluble form of FpoF that is characterized by a slow electron transfer from Fd_{red} onto F_{420}.

The existence of a Fd: F_{420} oxidoreductase, identified as soluble FpoF, surely contributes to the regeneration of Fd_{red} and to the survival of the *Ms. mazei* Δech mutant. Although, this cytoplasmic enzyme does not explain the relatively high reduction rate of CoM-S-S-CoB at

the expense of Fd_{red} observed in the membrane fraction of this mutant. Therefore, it is tempting to speculate that besides FpoF there is another still unknown protein able to channel electrons from Fd_{red} into the respiratory chain. Furthermore, it is important to note that the efficiency of energy conservation using Fd_{red} is higher compared to $F_{420}H_2$ because the membrane-bound Fd: CoM-S-S-CoB oxidoreductase system translocates more protons over the cytoplasmic membrane than the $F_{420}H_2$: CoM-S-S-CoB oxidoreductase system [11]. Hence, the cells have to avoid a major transfer of electrons from Fd_{red} to F_{420}, which is accomplished by the low activity of the Fd: F_{420} oxidoreductase. It is tempting to speculate that the enzyme acts as a valve to establish the equilibrium of different reducing equivalent concentrations, as well as in the transition of methanogenic pathways, e.g. from aceticlastic to methylotrophic growth.

MATERIALS AND METHODS

Strains, culture conditions and growth measurement

Ms. mazei Gö1 (DSM 7222) was used as parent strain for mutant generation and wildtype strain. Furthermore, a *Ms. mazei* mutant lacking Ech hydrogenase (*Ms. mazei* Δech) [28], as well as ΔfpoF and ΔfpoA-O mutant strains (generation described below) were used. In addition, *Ms. barkeri* (DSM 800^T) was used for cell lysate experiments. All strains were grown in DSM medium 120 with TMA (50 mM final concentration) or acetate (100 mM final concentration) as substrate. To monitor growth, 50 mL cultures were grown and samples taken at different time points. They were reduced with sodium dithionite and the optical density at 600 nm was determined using a Helios Epsilon spectrophotometer (Thermo Scientific, Germany). All growth experiments were performed with at least 4 independent cultures.

Resting cell suspension measurements

Mid-exponentially grown *Ms. mazei* wildtype and mutant strain cultures were harvested, washed once in stabilizing buffer (2 mM KH_2PO_4/K_2HPO_4, 2 mM $MgSO_4$, 20 mM NaCl, 200 mM sucrose, pH 6.8) and resuspended in the same buffer to yield a final protein content of 0.5 – 1 mg mL^{-1}. The cells starved at 37 ℃ for 30 min before methane formation was induced by adding 5 mM trimethylamine. If desired, the 100 % N_2 headspace was replaced by 100 % H_2. At various reaction time points, 50 µL of the headspace were injected into a gas chromatograph (GC-14A, Shimadzu, Kyoto, Japan) with N_2 as carrier gas. Methane was analyzed by a flame ionization detector and quantified by comparison with a standard curve.

Generation of mutant strains

Ms. mazei ΔfpoF and ΔfpoA-O were generated using homologous recombination as described by Metcalf [29]. For the creation of knockout vectors, fragments of the up- and downstream regions of the respective genes or gene clusters were amplified by PCR and cloned into the two multiple cloning sites of the pJK3 vector [29]. For the ΔfpoF knockout, a 1.2 kb fragment upstream of *mm0627 (fpoF)* was cloned into pJK3 using *Xho*I and *Eco*RV (using the primers 5'-CCGGCTCGAGTGCAATTAACATCTATTGTA-3' and 5'-CCTTGATATCTCAGTTACCTCCACTGCCT-3' for the generation of the upstream PCR fragment), and a 1.2 kb fragment downstream of *mm0627* was cloned into pJK3 using *Spe*I and *Not*I (using the primers 5'-GTAACTAGTCAGTCTGAAGTCCGAAACTT-3' and 5'-AGCGCGGCCGCGGAAAGTGGTCTACCTTA-3' for the generation of the downstream PCR fragment). The knockout vector was linearized with *Xho*I and transformed into *Ms. mazei* as described [30]. For the ΔfpoA-O knockout, a 1.2 kb fragment upstream of *mm2491 (fpoA)* was cloned into pJK3 using *Xho*I and *Hin*dIII (primers 5'-CCTTCTCGAGGCCCTCCAAGTCCTGCACCT-3' and 5'-CATGAAGCTTAGTGCAGCAATCTGAAATTGC-3'), and a 1.2 kb fragment downstream of *mm2479 (fpoO)* was cloned using *Sac*I and *Bcu*I (employing the primers 5'-AACGCTGCAGGAACACGTACACCCGCATTA-3' and 5'-TACTACTAGTCCTCAGTTGGACGTTTACTC-3'). The ΔfpoA-O knockout vector was linearized with *Bcu*I and transformed into *Ms. mazei*. After clonal separation on agar plates, the mutant cultures were confirmed by PCR. Gene-specific primer for *fpoD (mm2488)* and *fpoF (mm0627)* revealed the presence or absence of the respective genes in the mutant strains. In parallel, a control PCR with wildtype DNA or cell material was performed. Furthermore, primers specific for the puromycin resistance cassette (pac) were used to verify the presence of the pac cassette in the mutant genomes. The absence of the FpoF protein in the ΔfpoF mutant was also verified by Western blot and antibody detection with antibodies directed against FpoF. More information about these genes and proteins can be found in the database KEGG

(http://www.genome.jp/kegg/) using the above-mentioned locus number of the genes from *Ms. mazei* (MM_0627, MM_2488, MM_2479, MM_2491)

Preparation of proteins, antibodies, membranes and cofactors

CO dehydrogenase from *Moorella thermoacetica* was purified as described [31] with the modifications as described in [28]. Purification of ferredoxin from *Clostridium pasteurianum* was performed as outlined by [32] with replacement of the last two steps (crystallization, dialysis) by ultrafiltration. Heterodisulfide was synthesized as specified in [33], and cofactor F_{420} was purified and reduced as described by Abken et al. [34]. Cell lysate was obtained by anaerobically harvesting trimethylamine-grown cells and resuspending in buffer A (40 mM K_2HPO_4/KH_2PO_4, pH 7.0, 5 mM dithioerythritol, 1 µg mL^{-1} resazurin) containing desoxyribonuclease to yield a final protein content of about 5 mg mL^{-1}. *Ms. mazei* cells immediately lyse in buffer A due to an osmotic shock; *Ms. barkeri* cells were broken by French pressure treatment at 1000 psi. Subsequent membrane preparation of *Ms. mazei* was carried out as described in [28]. The cytoplasmic supernatant was ultracentrifuged twice (120 000 x g, 45 min) and only the top 60 % of the ultracentrifugation supernatant was defined as “membrane-free cytoplasm”.

For antibody production and enzyme assays, FpoF was heterologously produced in *Escherichia coli* DH5α. The *fpoF* gene (*mm0627*) was cloned into pASK-IBA3 (IBA, Göttingen, containing a C-terminal StrepII-Tag). The *fpoF* PCR fragment was generated with the primers 5'-ATGGTACGTCTCAAATGCCACCAAAGATTGC AGAAGTCATT-3' and 5'-ATGGTACGTCTCAGCGCTGACTGTTTCACTG CGGATTCCG-3'. It was digested with *Bsm*BI and cloned into the *Bsa*I sites of pASK-IBA3. The resulting construct was checked by sequencing (StarSEQ, Mainz, Germany). Expression was performed in 200 mL maximal induction (MI) medium ([35], 32 g L^{-1} trypton, 20 g L^{-1} yeast extract) containing M9 salts, 100 µM $CaCl_2$, 1 mM $MgSO_4$ and 1 µM $FeNH_4$ citrate. The cells were grown until an optical density of approximately 1 was reached, then protein production was induced with 200 ng mL^{-1} anhydrotetracyclin. Furthermore, 30 µM $FeNH_4$ citrate and 40 µg mL^{-1} riboflavin were added, the culture was flushed with N_2 and sealed with a rubber stopper to achieve anoxic conditions. After a 4 h induction period at 30° C with gentle shaking (50 rpm), the culture was harvested anaerobically (10 000 x g, 15 min). To facilitate lysis of the cells, the pellet was frozen and thawed, then resuspended in buffer W (150 mM Tris, pH 8.0, 5 mM dithioerythritol, 1 µg mL^{-1} resazurin) containing a small amount of lysozyme, desoxyribonuclease and 1 mL B-PER protein extraction solution (Thermo Fisher, Schwerte, Germany) per mL buffer W. After clarification of the lysate (25 000 x g, 15 min, 4° C), the recombinant protein was purified anaerobically in an anaerobic chamber (3 % H_2, 97 % N_2, Coy Laboratories, MI, USA) using Strep-Tactin sepharose as described by the manufacturer (IBA, Göttingen, Germany).

Antibodies were produced by Seqlab (Göttingen, Germany) with the 3-month protocol using rabbits for immunization. Horseradish peroxidase conjugated goat-anti-rabbit-IgG was purchased from Rockland Inc. (Gilbertsville, PA, USA).

Cofactor quantification

Non-heme iron was quantified as described by Landers & Sak [36]. Acid-labile sulfide was quantified photometrically at 670 nm by measuring the formation of methylene blue after the addition of N,N-dimethyl-p-phenylenediamine, with Na_2S as standard [37]. Flavin identity and content were determined by HPLC analysis (Knauer Smartline, Berlin, Germany) with a reverse-phase C-18 column (Varian Microsorb-MV, 250 mm x 4.6 mm) at a flow rate of 0.75 mL min^{-1} with a linear gradient of 0 – 100 % methanol in ammonium acetate buffer (50 mM, pH 6.0). Flavins were detected at 436 nm [12] with a retention time of 12.6 min for FAD and 14.9 min for FMN. Prior to loading onto the HPLC column, protein samples were treated with 5 % trichloroacetic acid for 15 min (Adams and Jia 2006), then precipitated protein was collected by centrifugation (8000 x g, 2 min) and protein-free supernatant was applied to the HPLC column. Peak areas were compared to an FAD standard curve between 0 – 20 µM.

Enzyme assays

All enzyme assays were performed in rubber-stoppered cuvettes or vials filled with buffer A with 100 % N_2 in the headspace unless indicated. $F_{420}H_2$: heterodisulfide oxidoreductase activity was determined in 600 µL buffer A using 10 µM $F_{420}H_2$, 20 µM CoM-S-S-CoB and 100 – 200 µg cell lysate, membrane fraction or cytoplasmic fraction. The increase of absorbance at 420 nm was recorded with a Jasco V-550 UV/Vis spectrophotometer (Gross-Umstadt, Germany), and a molar extinction coefficient of F_{420} of 40 mM^{-1} cm^{-1} was used for the calculation of

enzyme activity. For the measurement of F_{420} hydrogenase, heterodisulfide was omitted and the amount of cell lysate increased up to 500 µg. For the reverse reaction, 100 % H_2 in the headspace, 100 – 200 µg cell lysate and 10 µM F_{420} were used.

FpoF activity was determined by observing the change of absorbance at 420 nm caused by F_{420} reduction. The forward reaction ($Fd_{red} \rightarrow F_{420}$) was measured using 600 µL buffer A under a 5 % CO / 95 % atmosphere, 5 µg Fd, 50 µg CO dehydrogenase and 15 µM F_{420}. The initial substrate CO passes electrons to the *Moorella thermoacetica* CODH/ACS, which reduces *Clostridium pasteurianum* ferredoxin (Fd). Fd_{red} is then used by FpoF to reduce F_{420}. For K_M and v_{max} calculation, variable amounts of Fd (0.5 – 20 µg) and F_{420} (1.2 – 10 µM) were used, whereas the other components were used in concentrations as described above.

Western Blot

Prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), membrane proteins were solubilized in 1 % decylmaltoside for 3 h or overnight, then mixed with SDS-PAGE loading dye [38] and directly applied to the gel. Cytoplasmic proteins as well as FpoF were heated in SDS-PAGE loading dye for 5 min at 95 ℃ prior to loading onto the gel. To the gel 10 – 100 µg membrane or cytoplasmic protein fraction and 10 – 100 ng purified FpoF were applied. All proteins were separated in a 12.5 % Laemmli SDS-PAGE [38] and blotted onto a nitrocellulose membrane using a semi-dry blotting device (Biozym, Hessisch Oldendorf, Germany). The membrane was blocked in PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) containing 5 % milk powder for 1 h at room temperature. Then the membrane was washed (3 x 5 min with PBS) and the primary antibody directed against FpoF (rabbit-αFpoF, 2^{nd} bleeding) was applied in a 1:1000 dilution in PBS. This was followed by a washing step with PBST (PBS containing 0.05 % Tween 20, 3 x 10 min) and incubation with the secondary antibody (HRP-conjugated goat-anti-rabbit-IgG) in a 1:5000 dilution. The membrane was washed again with PBST (3 x 10 min), and detection was performed in 20 mL PBS containing 200 µL 4-chloro-1-naphthol (3 % w/v) and 20 µL H_2O_2 (30 %). Quantification was performed using a Canon CanoScan 4400F flatbed scanner (Canon, Krefeld, Germany) and Adobe Photoshop with the method outlined at http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html. Briefly, membranes were scanned in black and white, the colors inverted and the relative intensity (luminescence x occupied pixel) determined using the "histogram" option. A calibration curve was constructed using different amounts of FpoF, and the amount of FpoF estimated in membrane and cytoplasmic fractions.

Acknowledgements

We thank Elisabeth Schwab for technical assistance and Paul Schweiger for critical reading of the manuscript. Many thanks also go to Gunes Bender and Steve Ragsdale, Department of Biological Chemistry, University of Michigan Medical School for providing the CODH/ACS from *Moorella thermoacetica*. This work was supported by the Deutsche Forschungsgemeinschaft (grant De488/9-1).

REFERENCES

1. Deppenmeier U & Müller V (2008) Life close to the thermodynamic limit: how methanogenic archaea conserve energy. *Results Probl Cell Differ* **45**, 123-152.

2. Bäumer S, Murakami E, Brodersen J, Gottschalk G, Ragsdale SW & Deppenmeier U (1998) The $F_{420}H_2$:heterodisulfide oxidoreductase system from *Methanosarcina* species. 2-Hydroxyphenazine mediates electron transfer from $F_{420}H_2$ dehydrogenase to heterodisulfide reductase. *FEBS Lett* **428**, 295-298.

3. Bäumer S, Ide T, Jacobi C, Johann A, Gottschalk G & Deppenmeier U (2000) The $F_{420}H_2$ dehydrogenase from *Methanosarcina mazei* is a redox-driven proton pump closely related to NADH dehydrogenases. *J Biol Chem* **275**, 17968-17973.

4. Kulkarni G, Kridelbaugh DM, Guss AM & Metcalf WW (2009) Hydrogen is a preferred intermediate in the energy-conserving electron transport chain of Methanosarcina barkeri. *Proc Natl Acad Sci USA* **106**, 15915-15920.

5. Tebrommelstroet BWJ, Geerts WJ, Keltjens JT, Vanderdrift C & Vogels GD (1991) Purification and properties of 5,10-methylenetetrahydromethanopterin dehydrogenase and 5,10-methylenetetrahydromethanopterin reductase, 2 coenzyme-F_{420}-dependent enzymes, from *Methanosarcina barkeri*. *Biochim Biophys Acta* **1079**, 293-302.

6. Ma KS & Thauer RK (1990) N5, N10-methylenetetrahydromethanopterin reductase from *Methanosarcina barkeri*. *FEMS Microbiol Lett* **70**, 119-124.

7. Yamazaki S & Tsai L (1980) Purification and properties of 8-hydroxy-5-deazaflavin-dependent

$NADP^+$ reductase from *Methanococcus vannielii*. *J Biol Chem* **255**, 6462-6465.

8. Kunow J, Schwörer B, Stetter KO & Thauer RK (1993) A F_{420}-dependent NADP reductase in the extremely thermophilic sulfate-reducing *Archaeoglobus fulgidus*. *Arch Microbiol* **160**, 199-205.

9. Berk H & Thauer RK (1998) $F_{420}H_2$: NADP oxidoreductase from *Methanobacterium thermoautotrophicum*: identification of the encoding gene via functional overexpression in *Escherichia coli*. *FEBS Lett* **438**, 124-126.

10. Elias DA, Juck DF, Berry KA & Sparling R (2000) Purification of the $NADP^+$: F_{420} oxidoreductase of *Methanosphaera stadtmanae*. *Can J Microbiol* **46**, 998-1003.

11. Welte C, Krätzer C & Deppenmeier U (2010) Involvement of Ech hydrogenase in energy conservation of *Methanosarcina mazei*. *FEBS J* **277**, 3396-3403.

12. Brüggemann H, Falinski F & Deppenmeier U (2000) Structure of the $F_{420}H_2$:quinone oxidoreductase of *Archaeoglobus fulgidus*. Identification and overproduction of the $F_{420}H_2$-oxidizing subunit. *Eur J Biochem* **267**, 5810-5814.

13. Muth E, Mörschel E & Klein A (1987) Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from the archaebacterium *Methanococcus voltae*. *Eur J Biochem* **169**, 571-577.

14. Brodersen J, Bäumer S, Abken HJ, Gottschalk G & Deppenmeier U (1999) Inhibition of membrane-bound electron transport of the methanogenic archaeon *Methanosarcina mazei* Gö1 by diphenyleneiodonium. *Eur J Biochem* **259**, 218-224.

15. Alex LA, Reeve JN, Orme-Johnson WH & Walsh CT (1990) Cloning, sequence determination, and expression of the genes encoding the subunits of the Nickel-containing 8-hydroxy-5-deazaflavin reducing hydrogenase from *Methanobacterium thermoautotrophicum* ΔH. *Biochemistry* **29**, 7237-7244.

16. Fox JA, Livingston DJ, Orme-Johnson WH & Walsh CT (1987) 8-hydroxy-5-deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum* .1. Purification and characterization. *Biochemistry* **26**, 4219-4227.

17. Livingston DJ, Fox JA, Orme-Johnson WH & Walsh CT (1987) 8-hydroxy-5-deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum* .2. Kinetic and hydrogen-transfer studies. *Biochemistry* **26**, 4228-4237.

18. Jacobson FS, Daniels L, Fox JA, Walsh CT & Orme-Johnson WH (1982) Purification and properties of an 8-hydroxy-5-deazaflavin reducing hydrogenase from *Methanobacterium thermoautotrophicum*. *J Biol Chem* **257**, 3385-3388.

19. Hovey R, Lentes S, Ehrenreich A, Salmon K, Saba K, Gottschalk G, Gunsalus RP & Deppenmeier U (2005) DNA microarray analysis of *Methanosarcina mazei* Gö1 reveals adaptation to different methanogenic substrates. *Mol Genet Genomics* **273**, 225-239.

20. Michel R, Massanz C, Kostka S, Richter M & Fiebig K (1995) Biochemical characterization of the 8-hydroxy-5-deazaflavin-reactive hydrogenase from *Methanosarcina barkeri* Fusaro. *Eur J Biochem* **233**, 727-735.

21. Guss AM, Mukhopadhyay B, Zhang JK & Metcalf WW (2005) Genetic analysis of *mch* mutants in two *Methanosarcina* species demonstrates multiple roles for the methanopterin-dependent C_1 oxidation/reduction pathway and differences in H_2 metabolism between closely related species. *Mol Microbiol* **55**, 1671-1680.

22. Guss AM, Kulkarni G & Metcalf WW (2009) Differences in hydrogenase gene expression between *Methanosarcina acetivorans* and *Methanosarcina barkeri*. *J Bacteriol* **191**, 2826-2833.

23. Rohlin L & Gunsalus RP (2010) Carbon-dependent control of electron transfer and central carbon pathway genes for methane biosynthesis in the archaean *Methanosarcina acetivorans* strain C2A. *BMC Microbiol* **10**, 62.

24. Zinder SH & Mah RA (1979) Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. *Appl Environ Microbiol* **38**, 996-1008.

25. Deppenmeier U (1991) Identifizierung und Charakterisierung membrangebundener protonentranslozierender Redox-Systeme in methanogenen Bakterien. PhD thesis, University of Göttingen.

26. Allen MA, Lauro FM, Williams TJ, Burg D, Siddiqui KS, De Francisci D, Chong KWY, Pilak O, Chew HH, De Maere MZ, Ting L, Katrib M, Ng C, Sowers KR, Galperin MY, Anderson IJ, Ivanova N, Dalin E, Martinez M, Lapidus A, Hauser L, Land M, Thomas T & Cavicchioli R (2009) The genome sequence of the psychrophilic archaeon *Methanococcoides burtonii*: the role of genome evolution in cold adaptation. *ISME J* **3**, 1012-1035.

27. Franzmann PD, Springer N, Ludwig W, Demacario EC & Rohde M (1992) A methanogenic archaeon from Ace Lake, Antarctica - *Methanococcoides burtonii* sp. nov. *Systematic and Applied Microbiology* **15**, 573-581.

28. Welte C, Kallnik V, Grapp M, Bender G, Ragsdale S & Deppenmeier U (2010) Function of

Ech hydrogenase in ferredoxin-dependent, membrane-bound electron transport in *Methanosarcina mazei*. *J Bacteriol* **192**, 674-678.
29. Metcalf WW, Zhang JK, Apolinario E, Sowers KR & Wolfe RS (1997) A genetic system for archaea of the genus *Methanosarcina*: Liposome-mediated transformation and construction of shuttle vectors. *Proc Natl Acad Sci USA* **94**, 2626-2631.
30. Ehlers C, Weidenbach K, Veit K, Deppenmeier U, Metcalf WW & Schmitz RA (2005) Development of genetic methods and construction of a chromosomal glnK1 mutant in *Methanosarcina mazei* strain Gö1. *Mol Genet Genomics* **273**, 290-298.
31. Ragsdale SW, Ljungdahl LG & Der Vartanian DV (1983) Isolation of carbon monoxide dehydrogenase from *Acetobacterium woodii* and comparison of its properties with those of the *Clostridium thermoaceticum* enzyme. *J Bacteriol* **155**, 1224-1237.
32. Mortenson LE (1964) Purification and analysis of ferredoxin from *Clostridium pasteurianum*. *Biochim Biophys Acta* **81**, 71-77.
33. Ellermann J, Hedderich R, Bocher R & Thauer RK (1988) The final step in methane formation. Investigations with highly purified methyl-CoM reductase (component C) from *Methanobacterium thermoautotrophicum* (strain Marburg). *Eur J Biochem* **172**, 669-677.
34. Abken HJ, Tietze M, Brodersen J, Bäumer S, Beifuss U & Deppenmeier U (1998) Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Gö1. *J Bacteriol* **180**, 2027-2032.
35. Mott JE, Grant RA, Ho YS & Platt T (1985) Maximizing gene expression from plasmid vectors containing the Lambda-Pl promoter - strategies for overproducing transcription termination factor Rho. *Proc Natl Acad Sci USA* **82**, 88-92.
36. Landers JW & Zak B (1958) Determination of serum copper and iron in single small sample. *Tech Bull Regist Med Technol* **28**, 98-100.
37. Beinert H (1983) Semi-micro methods for analysis of labile sulfide and of labile sulphide plus sulfane sulfur in unusually stable iron–sulfur proteins. *Anal Biochem* **131**, 373-378.
38. Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* **227**, 680-685.

Supplementary Material

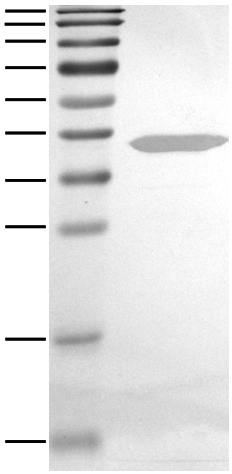

Figure S1: SDS-PAGE of recombinant FpoF. 1 µg FpoF was loaded onto a 12.5 % SDS gel. Molecular mass standard (top to bottom): 170 kDa, 130 kDa, 95 kDa, 72 kDa, 55 kDa, 43 kDa, 34 kDa, 26 kDa, 17 kDa, 10 kDa.

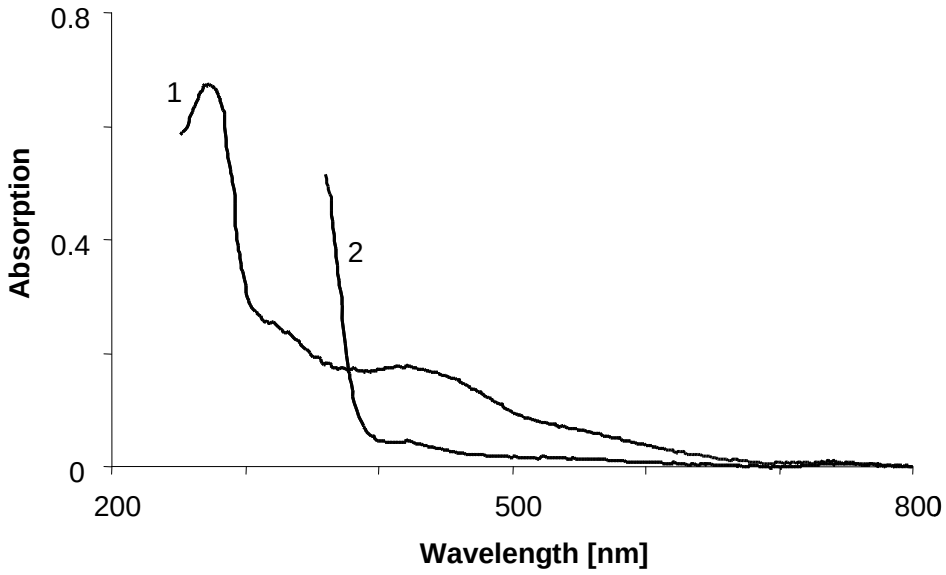

Figure S2: UV/Visible spectra of recombinant FpoF. The cuvette contained 1 mg mL^{-1} FpoF, 320 µM H_2O_2 for oxidation and a few grains of sodium dithionite for reduction**.**

Chapter 5
Membrane-bound electron transport in *Methanosaeta thermophila*

Energy conservation in *Methanosarcina* sp. is well understood, and useful tools were found for the investigation of all membrane-bound heterodisulfide oxidoreductase systems. Stoichiometries of translocated H^+ or Na^+ per 2 e^- could be established for all known oxidoreductase systems of *Ms. mazei*, and only few unanswered questions remain. In contrast, there is essentially nothing known about the energy conserving mechanisms in *Methanosaeta* sp. although this genus is unique in its obligate aceticlastic growth. Together with *Methanosarcina* sp., these organisms form the major group contributing to biogenic methane emission. At high acetate concentrations the faster growing *Methanosarcina* sp. outcompete *Methanosaeta* sp., but at low acetate concentrations (< 1 mM, Jetten *et al.*, 1992b) *Methanosaeta* sp. prevail. Faster growth, easier cultivation and the long-known genome sequences of many *Methanosarcina* sp. has biased the research on energy conservation towards members of the *Methanosarcinaceae* although members of the *Methanosaetaceae* are of no less importance. The deciphering of the first *Methanosaeta* genome in 2007 (Smith and Ingram-Smith, 2007) could have unravelled the functioning of *Methanosaeta* energy conservation, but the genome did not contain genes encoding known electron-input complexes used in the respiratory systems of other aceticlastic methanogens: the genome neither contained genes coding for Ech hydrogenase or the Rnf complex. Other ferredoxin-interacting, membrane-bound enzymes from methanogens are not known. The investigations presented in this paper focused on two issues: (i) Is a membrane-bound heterodisulfide oxidoreductase system present in *Methanosaeta thermophila*? (ii) What is the electron donor to the *Mt. thermophila* respiratory chain?

As a result, ferredoxin was the only electron donor that interacted with the membrane. Reduced ferredoxin was the electron donor to a heterodisulfide oxidoreductase system and high activities of the ferredoxin: heterodisulfide oxidoreductase could be observed. The lack of Ech hydrogenase and the Rnf complex indicates that a novel type of ferredoxin-interacting enzyme is involved.

Welte and Deppenmeier (2011) *J Bacteriol* **193**:2868-2870

Membrane-bound electron transport in *Methanosaeta thermophila*

Cornelia Welte and Uwe Deppenmeier

Institute of Microbiology and Biotechnology, University of Bonn, Meckenheimer Allee 168, 53115 Bonn, Germany

The obligate aceticlastic methanogen *Methanosaeta thermophila* uses a membrane-bound ferredoxin: heterodisulfide oxidoreductase system for energy conservation. We propose that the system is composed of a truncated form of the $F_{420}H_2$ dehydrogenase, methanophenazine and the heterodisulfide reductase. Hence, the electron transport chain is distinct from those of well studied *Methanosarcina* species.

Biogenic methane production is dominated by Methanoarchaea of the genera *Methanosarcina* (*Ms*.) and *Methanosaeta* (*Mt*.) that grow on acetate (6). Interestingly, *Methanosaeta* species can use only acetate as substrate and are therefore obligate aceticlastic methanogens. Members of this genus are of special importance for the productivity of biogas plants, especially for reactor performance and stability at low acetate concentrations. To optimize biomethanation, it is necessary to acquire a comprehensive understanding of the biochemistry of acetate-dependent methanogenesis. Energy conservation in *Methanosaeta* sp. is not well understood, and even the sequencing of the *Mt. thermophila* genome (13) did not unravel its mechanism. Comparative genomics indicated that the core methanogenic pathway, the breakdown of acetyl-CoA to methane, is obviously well conserved in *Mt. thermophila*. It can be concluded that acetate is activated by acetyl-CoA synthetases and the resulting acetyl-CoA serves as substrate for a CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) that oxidizes the carbonyl group to CO_2 and reduces ferredoxin. The methyl group is first transferred to tetrahydromethanopterin, and then to coenzyme M (CoM, 2-mercaptoethanesulfonate) by the action of a membrane-bound Na^+ translocating methyltransferase. Methyl-CoM is oxidatively coupled to coenzyme B (CoB, N-7-mercaptoheptanoyl-L-threonine phosphate) with the heterodisulfide CoM-S-S-CoB and methane as end products (5, 15). In contrast, the composition of the *Mt. thermophila* respiratory chain and the mode of energy conservation remained largely unknown. Evidence was only found for the presence of the reduced ferredoxin (Fd_{red}) forming CODH/ACS and the heterodisulfide reductase (1, 13, 14). In *Methanosarcina* sp. a ferredoxin: heterodisulfide oxidoreductase is used for energy conservation in acetate metabolism. *Ms. mazei* and *Ms. barkeri* employ the Ech hydrogenase for H_2 production from Fd_{red} and the H_2-uptake hydrogenase (Vho) that finally reduces methanophenazine, the electron donor for the heterodisulfide reductase (HdrDE). In *Ms. acetivorans*, Ech hydrogenase is absent, and instead the Rnf complex is proposed to be responsible for Fd_{red} oxidation (6). Surprisingly, the *Mt. thermophila* genome does not contain genes coding for either hydrogenases or an Rnf complex (13). If Fd_{red} serves as electron donor for the respiratory chain, the presence of a novel oxidoreductase must be postulated.

To investigate the electron transport processes in *Mt. thermophila*, we isolated cytoplasmic membranes from *Mt. thermophila* DSM6194 as described (17) with cell disruption by French pressure treatment (1000 psi). Enzyme assays were carried out at 55° C (optimal growth temperature) (3, 17). Benzyl viologen-dependent heterodisulfide reductase activity was high with 878 ± 90 mU mg^{-1} membrane protein (Table 1) and was comparable to activities found in *Methanosarcina* sp. (3) (Figure 1). Hence, it is tempting to speculate that a membrane-bound heterodisulfide oxidoreductase system is used for energy conservation in *Mt. thermophila*, with a so far unidentified enzyme system that channels electrons into the respiratory chain. Genes encoding the $F_{420}H_2$ dehydrogenase (Fpo) were identified in the genome of *Mt. thermophila* and this protein is therefore a candidate for electron input into the respiratory chain. Fpo is usually involved in methylotrophic methanogenesis and oxidizes $F_{420}H_2$ that is formed in the methanogenic pathway of *Methanosarcina* sp. The *Methanosarcina* core enzyme FpoA-O is highly homologous to NADH dehydrogenase I from bacteria and eukarya. However, the reduced cofactor oxidizing subunits from $F_{420}H_2$ dehydrogenases and NADH dehydrogenases are not homologous. The corresponding module of the bacterial and eukaryotic enzymes is made from subunits NuoEFG. In contrast, the oxidation of reduced cofactor F_{420} is catalyzed by subunit

Table 1: Activities of membrane-bound oxidoreductases in *Mt. thermophila*.

Enzyme [1] (system)	electron donor	electron acceptor	reduction rate of electron acceptor (nmol min^{-1} mg^{-1})
$F_{420}H_2$ dehydrogenase	$F_{420}H_2$	metronidazole	< 1
Hydrogenase	H_2	methyl viologen	< 1
Heterodisulfide reductase	benzyl viologen	CoM-S-S-CoB	878 ± 90
$F_{420}H_2$: heterodisulfide oxidoreductase	$F_{420}H_2$	CoM-S-S-CoB	< 1
H_2: heterodisulfide oxidoreductase	H_2	CoM-S-S-CoB	< 1
Fd: heterodisulfide oxidoreductase	Fd_{red}	CoM-S-S-CoB	470 ± 44
NADH: heterodisulfide oxidoreductase	NADH	CoM-S-S-CoB	< 1
NADPH: heterodisulfide oxidoreductase	NADPH	CoM-S-S-CoB	< 1

1) Assay conditions were according to (3, 17).

FpoF of the $F_{420}H_2$ dehydrogenase (4) Interestingly, the *Mt. thermophila* genome only codes for an incomplete $F_{420}H_2$ dehydrogenase (FpoA-O) that lacks FpoF and thus should be unable to oxidize $F_{420}H_2$ as shown for the *Ms. mazei* Δ*fpoF* mutant (16). Nevertheless, substantial quantities of F_{420} can be found in *Mt. thermophila* cells (9), so the $F_{420}H_2$ oxidizing reactivity of the membranes was determined. As expected we could neither detect $F_{420}H_2$: heterodisulfide oxidoreductase activity nor $F_{420}H_2$ dehydrogenase activity (Table 1). These findings show that energy conservation is not dependent on F_{420}. Also NAD(P)H did not serve as electron donor for heterodisulfide reduction in *Mt. thermophila* (Table 1). Many methanogens rely on hydrogen as electron donor and/or obligate intermediate in the oxidation of other reducing equivalents (Fd_{red}/$F_{420}H_2$). For this purpose, some methanogens make use of a cytoplasmic F_{420} reducing hydrogenase (Frh) that can oxidize $F_{420}H_2$ with concomitant H_2 production and then use the membrane-bound Vho hydrogenase: heterodisulfide oxidoreductase to conserve energy (10, 16). *Mt. thermophila* does not possess genes coding for Frh or Vho, and indeed there was no hydrogenase activity or hydrogen: heterodisulfide oxidoreductase activity in *Mt. thermophila* membranes (Table 1). So an involvement of hydrogen or a hydrogen cycling mechanism for energy conservation in *Mt. thermophila* can be excluded. For the investigation of the Fd: heterodisulfide oxidoreductase in *Mt. thermophila*, a *Ms. mazei* ferredoxin – MM1619 – was employed. The gene *mm1619* was cloned into pPR-IBA1 using *Bsa*I restriction sites (primers 5'-ATGGTAGGTCTCAAATGCCAGCAATAGTTAACGCAGATGAA-3' and 5'-ATGGTAGGTCTCAGCGCTTTCCGTTACTTTAATTGCCTGGTTC-3') and the recombinant protein produced in *Escherichia coli* BL21 (DE3) (8, 12) and purified anaerobically (16). When ferredoxin MM1619 was reduced with the thermophilic *Moorella thermoacetica* CODH/ACS and incubated with *Mt. thermophila* membranes, heterodisulfide reduction was observed. This reaction was strictly dependent on ferredoxin and proceeded with a velocity of 470 ± 44 mU mg^{-1} membrane protein (Table 1). In comparison to experiments performed with membranes isolated from acetate-grown *Ms. mazei* (unpublished results), this reaction was two- to three-fold faster in *Mt. thermophila* than in *Ms. mazei*. These experiments elucidate the identity of the electron donor to the *Methanosaeta* respiratory chain as ferredoxin (Figure 1). Our current working hypothesis is that in *Mt. thermophila* the energy conserving system is a Fd: heterodisulfide oxidoreductase that comprises the Fpo complex (without FpoF) and the heterodisulfide reductase, both of which are probably able to translocated H^+ or Na^+ across the cytoplasmic membrane (2, 7). The "head-less" Fpo complex does not interact with $F_{420}H_2$ but it is tempting to speculate that iron-sulfur clusters in the FpoB or FpoI subunits directly accept electrons from Fd_{red}. In addition, it is evident that the membrane-bound methyltransferase contributes to the maintenance of the electrochemical ion gradient. An A_1A_O ATP synthase finally takes advantage of the ion motive force and produces ATP from ADP + P_i (11) In the light of the discussion about energy conserving systems in aceticlastic methanogens, it is important to note that a Fd:

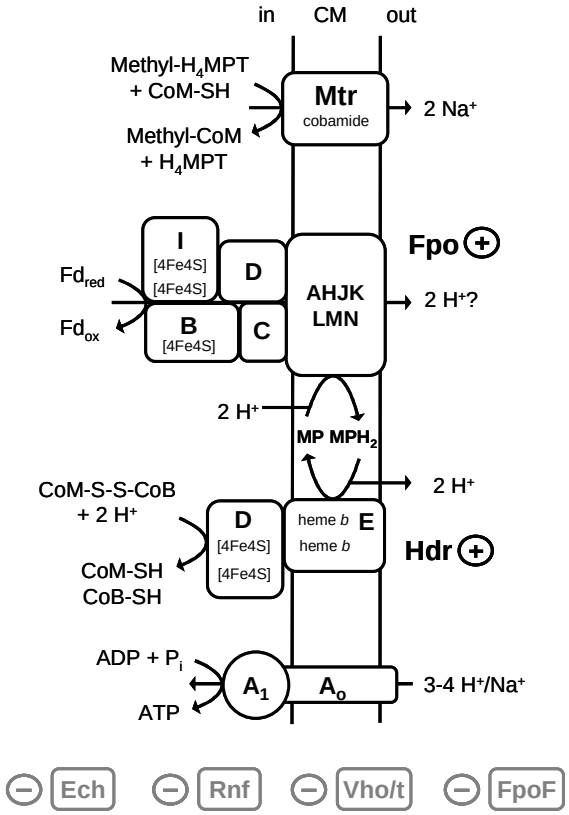

Figure 1: Putative model of energy conserving electron transfer reactions in *Mt. thermophila*. A_1A_O, A_1A_O ATP synthase; CM, cytoplasmic membrane; Ech, Ech hydrogenase; Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin; FpoABCDHIJKLMN, subunits A-N of the $F_{420}H_2$ dehydrogenase; FpoF, F-subunit of the $F_{420}H_2$ dehydrogenase; H_4MPT, tetrahydromethanopterin; HdrDE, heterodisulfide reductase subunits D and E; Mtr, methyltransferase; MP, methanophenazine; MPH_2, reduced methanophenazine; Rnf, Rnf complex; Vho/t, viologen-reducing hydrogenase one/two; (+), present in *Mt. thermophila*; (-), not present in *Mt. thermophila*.

heterodisulfide oxidoreductase activity was also found in the membrane fraction of a *Ms. mazei* Δech mutant (17). The organism also contains the Fpo complex and it was shown that subunit FpoF is in part located in the cytoplasm indicating that the Fpo complex is not always completely covered by FpoF. This situation resembles the electron transport system of *Mt. thermophila* and it is tempting to speculate that the Fpo complex (without FpoF) of *Ms. mazei* is also able to catalyze the reduction of Fd_{red} thereby channelling electron directly into the respiratory chain.

We thank Elisabeth Schwab for technical assistance and Paul Schweiger for critical reading of the manuscript. Many thanks also go to Gunes Bender and Steve Ragsdale, Department of Biological Chemistry, University of Michigan Medical School for providing the CODH/ACS from *Moorella thermoacetica*. This work was supported by the Deutsche Forschungsgemeinschaft (grant De488/9-1).

1. **Allen, G. W. J., and S. H. Zinder.** 1996. Methanogenesis from acetate by cell-free extracts of the thermophilic acetotrophic methanogen *Methanothrix thermophila* CALS-1. Arch. Microbiol. **166:**275-281.

2. **Bäumer, S., T. Ide, C. Jacobi, A. Johann, G. Gottschalk, and U. Deppenmeier.** 2000. The $F_{420}H_2$ dehydrogenase from *Methanosarcina mazei* is a redox-driven proton pump closelyrelated to NADH dehydrogenases. J. Biol. Chem. **275:**17968-17973.

3. **Brodersen, J., S. Bäumer, H. J. Abken, G. Gottschalk, and U. Deppenmeier.** 1999. Inhibition of membrane-bound electron transport of the methanogenic archaeon *Methanosarcina mazei* Gö1 by diphenyleneiodonium. Eur. J. Biochem. **259:**218-224.

4. **Brüggemann, H., F. Falinski, and U. Deppenmeier.** 2000. Structure of the $F_{420}H_2$: quinone oxidoreductase of *Archaeoglobus fulgidus* - Identification and overproduction of the $F_{420}H_2$-oxidizing subunit. Eur. J. Biochem. **267:**5810-5814.

5. **Deppenmeier, U.** 2002. The unique biochemistry of methanogenesis. Prog. Nucleic Acid Res. Mol. Biol. **71:**223-283.

6. **Ferry, J. G., and D. J. Lessner.** 2008. Methanogenesis in marine sediments. Ann. N. Y. Acad. Sci. **1125:**147-157.
7. **Ide, T., S. Bäumer, and U. Deppenmeier.** 1999. Energy conservation by the H_2:heterodisulfide oxidoreductase from *Methanosarcina mazei* Gö1: identification of two proton-translocating segments. J. Bacteriol. **181:**4076-4080.
8. **Jeoung, J. H., and H. Dobbek.** 2007. Carbon dioxide activation at the Ni,Fe-cluster of anaerobic carbon monoxide dehydrogenase. Science **318:**1461-1464.
9. **Kamagata, Y., H. Kawasaki, H. Oyaizu, K. Nakamura, E. Mikami, G. Endo, Y. Koga, and K. Yamasato.** 1992. Characterization of 3 thermophilic strains of *Methanothrix (Methanosaeta) thermophila* sp. nov. and rejection of *Methanothrix (Methanosaeta) thermoacetophila*. Int. J. Syst. Evol. Microbiol. **42:**463-468.
10. **Kulkarni, G., D. M. Kridelbaugh, A. M. Guss, and W. W. Metcalf.** 2009. Hydrogen is a preferred intermediate in the energy-conserving electron transport chain of *Methanosarcina barkeri*. Proc. Natl. Acad. Sci. USA **106:**15915-15920.
11. **Lewalter, K., and V. Müller.** 2006. Bioenergetics of archaea: Ancient energy conserving mechanisms developed in the early history of life. Biochim. Biophys. Acta **1757:**437-445.
12. **Nakamura, M., K. Saeki, and Y. Takahashi.** 1999. Hyperproduction of recombinant ferredoxins in *Escherichia coli* by coexpression of the ORF1-ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 gene cluster. J. Biochem. **126:**10-18.
13. **Smith, K. S., and C. Ingram-Smith.** 2007. *Methanosaeta*, the forgotten methanogen? Trends Microbiol. **15:**150-155.
14. **Terlesky, K. C., and J. G. Ferry.** 1988. Ferredoxin requirement for electron transport from the carbon monoxide dehydrogenase complex to a membrane-bound hydrogenase in acetate-grown *Methanosarcina thermophila*. J. Biol. Chem. **263:**4075-9.
15. **Thauer, R. K., A. K. Kaster, H. Seedorf, W. Buckel, and R. Hedderich.** 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. **6:**579-591.
16. **Welte, C., and U. Deppenmeier.** 2011. Re-evaluation of the function of the F_{420} dehydrogenase in electron transport in *Methanosarcina mazei*. FEBS J, in press.
17. **Welte, C., V. Kallnik, M. Grapp, G. Bender, S. Ragsdale, and U. Deppenmeier.** 2010. Function of Ech hydrogenase in ferredoxin-dependent, membrane-bound electron transport in *Methanosarcina mazei*. J. Bacteriol. **192:**674-678.

Chapter 6
Proton translocation in methanogens

Methanogenic bioenergetics has been investigated for over 20 years now. However, a comprehensive manual for the investigation of electron transport processes and the measurement of proton translocation has not been published. This publication compiles and updates protocols for the measurement of enzymatic activities involved in methanogenic energy conservation and facilitates comparison and accomplishments of the respective experiments. Furthermore, many cofactors needed in this field of research are not commercially available and have to be synthesized *de novo* or from commercial precursors. For some of the cofactors fractional descriptions for the synthesis existed (e.g. CoM-S-S-CoB) whereas for others (e.g. F_{420}) a comprehensive explanation of the isolation or synthesis was missing.

In the course of this work the protocols that are described below were refined and in part newly established for the investigation of the ferredoxin: heterodisulfide oxidoreductase of *Ms. mazei*. The previous protocols do not allow continuous measurement of the electron flow from reduced ferredoxin to the heterodisulfide. Furthermore, the analysis of proton translocation by pH measurement as performed for other oxidoreductase systems in *Ms. mazei* was not applicable. The results presented in the previous chapters are based on the methods and protocols described below. These newly developed approachs combine different test systems and allow dependable conclusions about ion translocation of the ferredoxin: heterodisulfide oxidoreductase system of *Ms. mazei* and other methanogenic archaea.

Welte and Deppenmeier (2011) *Methods Enzymol* **494**:257-280

Proton translocation in methanogens

Cornelia Welte and Uwe Deppenmeier

Institute of Microbiology and Biotechnology, University of Bonn, Meckenheimer Allee 168, 53115 Bonn, Germany

Methanogenic archaea of the genus *Methanosarcina* possess a unique type of metabolism because they use H_2 + CO_2, methylated C_1-compounds or acetate as energy and carbon source for growth. The process of methanogenesis is fundamental for the global carbon cycle and represents the terminal step in the anaerobic breakdown of organic matter in freshwater sediments. Moreover, methane is an important greenhouse gas that directly contributes to climate changes and global warming. *Methanosarcina* species convert the aforementioned substrates to CH_4 via the CO_2-reducing, the methylotrophic or the aceticlastic pathway. All methanogenic processes finally result in the oxidation of two thiol-containing cofactors (HS-CoM and HS- CoM) leading to the formation of the so-called heterodisulfide (CoM-S-S-CoB) that contains an inter-molecular disulfide bridge. This molecule functions as the terminal electron acceptor of a branched respiratory chain. Molecular hydrogen, reduced coenzyme F_{420} or reduced ferredoxin are used as electron donors. The key enzymes of the respiratory chain (Ech hydrogenase, F_{420}-nonreducing hydrogenase, $F_{420}H_2$ dehydrogenase and heterodisulfide reductase) couple the redox reactions to proton translocation across the cytoplasmic membrane. The resulting electrochemical proton gradient is the driving force for ATP synthesis. Here we describe the methods and techniques how to analyse electron transfer reactions, the process of proton translocation and the formation of ATP.

1. Introduction

Methanogenic archaea produce methane as the major metabolic end product and combine the pathway of methanogenesis with a unique energy-conserving system. Biological methane formation is one of the most important processes for the maintenance of the global carbon flux because these organisms perform the terminal step in the mineralization of organic material in many anaerobic environments. In addition, methane is an important greenhouse gas that significantly contributes to climate changes and global warming. In this context it is important to note that the atmospheric methane concentration increased more than 2.5-fold during the past 150 years due to anthropogenic effects including expanded cultivation of rice and ruminant life stock (Khalil and Rasmussen, 1994; Jain *et al.*, 2004). Thus, it is essential to consider the future atmospheric CH_4 budgets for the stabilization of greenhouse gas concentrations. On the other hand, the formation of biogenic methane as part of the so-called biogas is of great economical importance. In recent decades, the combustion of biogas, produced by fermenting biomass, to generate electricity in combined heat and power stations has largely expanded (Kashyap *et al.*, 2003).

Most methanogenic archaea can use H_2 + CO_2 as substrates. However, only methylotrophic methanogens (e.g. the genus *Methanosarcina*) are able to produce methane from methylated C_1-compounds such as methanol, methylamines, or methylthiols. Furthermore, only the genera *Methanosarcina (Ms.)* and *Methanosaeta* are able to use acetic acid for methanogenesis and growth (Boone *et al.*, 1993). Acetate is converted to methane and CO_2 and creates approximately 70 % of the biologically produced methane (Ferry and Lessner, 2008). All methanogenic substrates are formed by a complex degradation processes of organic matter as catalyzed by fermentative and synthrophic bacteria (McInerney *et al.*, 2009). The substrates are converted to CH_4 via the CO_2-reducing, the methylotrophic or the aceticlastic pathway of methanogenesis (Deppenmeier, 2002b). The biochemistry of methanogenesis clearly indicates that there is no site for ATP regeneration by substrate level phosphorylation. Therefore, it has been assumed for quite some time that membrane-bound electron transport might be coupled to ion translocation and that the ion motive force generated might be used for ATP synthesis (Thauer *et al.*, 1977).

The process of methanogenesis is very complex and involves many unusual enzymes

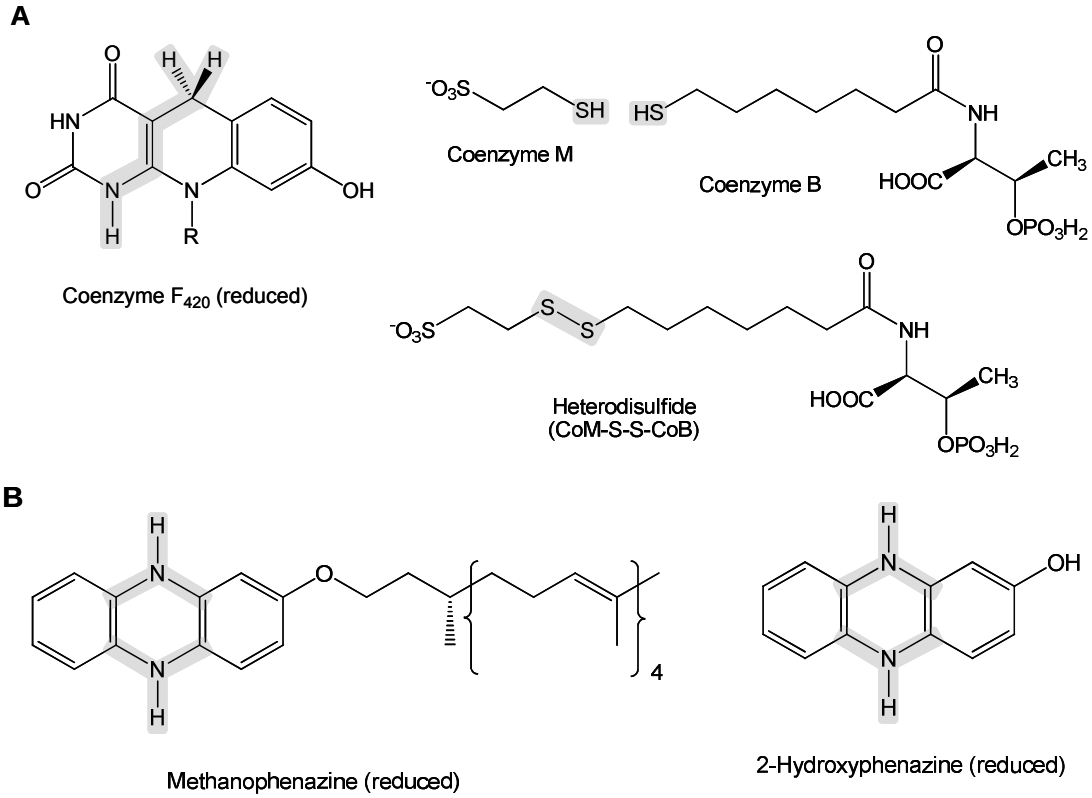

Figure 1: Electron carriers in *Ms. mazei.*

and cofactors (Wolfe, 1985; Thauer, 1998; Ferry, 1999; Deppenmeier and Müller, 2008; Thauer *et al.*, 2008). However, all catabolic processes ultimately lead to the formation of methyl-2-mercaptoethanesulfonate (methyl-S-CoM) (Fig. 1), which is then reduced to CH_4 by the catalytic activity of the methyl-S-CoM reductase. The enzyme uses N-7-mercaptoheptanoyl-L-threonine phosphate (HS-CoB) (Fig. 1) as the electron donor; hence the final product of this reaction is the heterodisulfide of HS-CoM and HS-CoB (CoM-S-S-CoB). This compound functions as electron acceptor of the methanogenic respiratory chain (Fig. 2). Molecular hydrogen, reduced coenzyme F_{420} (Fig. 1) or reduced ferredoxin are used as electron donors. The redox reactions, catalyzed by the membrane-bound electron transport chains, are coupled to proton translocation across the cytoplasmic membrane (Fig. 2). The resulting electrochemical proton gradient is the driving force for ATP synthesis as catalyzed by an A_1A_O-type ATP synthase (Pisa *et al.*, 2007; Deppenmeier and Müller, 2008).

2. Analysis of membrane-bound electron transport in *Ms. mazei*

The question as to the mechanism of ATP synthesis in methanogenic archaea has been investigated in whole cells (Blaut and Gottschalk, 1984; Blaut *et al.*, 1987; Peinemann *et al.*, 1988) or in protoplasts (Peinemann *et al.*, 1989). The results obtained were in accordance with a chemiosmotic mechanism of ATP synthesis. Further details of the energy metabolism were obtained when reduction of CoM-S-S-CoB with H_2 was studied using subcellular membrane preparations of *Ms. mazei* (Deppenmeier *et al.*, 1992).

2.1 Preparation of cytoplasmic membranes

In *Ms. mazei* all enzymes involved in energy transduction are tightly membrane-bound (Fig. 2). Hence, for the analysis of electron transfer events the preparation of cytoplasmic membranes is necessary. Washing steps of the membrane fraction are performed to remove all soluble cellular components.

Procedure: Ms. mazei is grown anaerobically at 37° C on the media described previously, supplemented with 1g/l of sodium acetate (Hippe *et al.*, 1979). The cultures are typically grown with trimethyamine as substrate (50 mM final concentration; doubling time 7.5 h). Other carbon and energy sources can be used at the following final concentrations: methanol (150 mM, doubling time 13 h), dimethylamine (100 mM), monomethylamine (150 mM) or sodium acetate (100 mM, doubling time 28 h).

Subsequent preparation steps are performed anaerobically in an anaerobic chamber (Coy

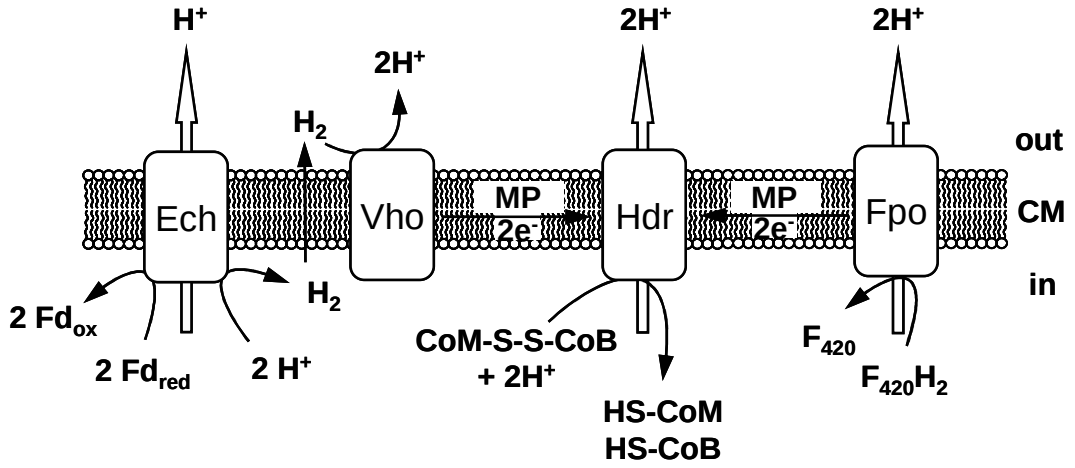

Figure 2: Membrane-bound electron transfer system of *Ms. mazei.* Ech, Echhydrogenase; Vho, F_{420}-nonreducing hydrogenase; Fpo, $F_{420}H_2$ dehydrogenase; Hdr, heterodisulfide reductase; MP, methanophenazine.

laboratory products, US) under a 97 % N_2 / 3 % H_2 atmosphere. Exponentially grown cells are filled in gas-tight centrifugation bottles (500 ml) and harvested by centrifugation (3000 x g, 30 min). The cell pellet is resuspended in a small volume 40 mM potassium phosphate (K-phosphate) buffer, pH 7.0, 5 mM dithioerythritol and 1 µg mL^{-1} resazurin. In the course of resuspension the cells lyse because the protein cell wall is not stabile in this buffer. After incubated with DNase I for 30 min at 4° C, the cell lysate is ultracentrifuged (90 min, 150,000 x g) and the supernatant is carefully removed and stored at –20° C (e.g. for a subsequent isolation of coenzyme F_{420}). The membrane pellet is homogenized in 20 mM K-phosphate buffer (pH 7.0) containing 20 mM $MgSO_4$, 500 mM sucrose, 5 mM dithioerythritol and 1 µg mL^{-1} resazurin. The ultracentrifugation step is repeated, the supernatant discarded and the membrane pellet again homogenized in the previously mentioned buffer (300 µL per L culture). The membrane fraction is finally checked for remaining contamination of soluble compounds of the cells by measuring the activity of the acetyl-CoA synthetase, which is only present in the cytoplasm of *Ms. mazei*.

To assay for acetyl-CoA synthetase activity an anaerobic rubber-stoppered glass cuvette containing 700 µL K-phosphate buffer (40 mM, pH 7.0, 5 mM dithioerythritol, 1 µg mL^{-1} resazurin) is flushed with 5 % CO / 95 % N_2. Methyl viologen is added to a final concentration of 5 mM (ε_{604}= 13.6 cm^{-1} x mM^{-1}). Upon addition of the prepared washed membrane fraction, no activity should occur if the membrane fraction is indeed cytoplasm-free.

2.2 Analysis of the H_2-dependent electron transport chain

The source of reducing equivalents necessary for the reduction of CoM-S-S-CoB depends on the growth substrate. If molecular hydrogen is present, a membrane-bound F_{420}-nonreducing hydrogenase channels electrons via b-type cytochromes to the heterodisulfide reductase, which reduces the terminal electron donor (Fig. 2) (Deppenmeier *et al.*, 1992). This electron transport system was referred to as H_2:heterodisulfide oxidoreductase. Later on it was found that the electron transport between the enzymes is mediated by the novel redox active cofactor methanophenazine (Fig. 1, 2) (Abken *et al.*, 1998; Beifuss and Tietze, 2000; Beifuss and Tietze, 2005). With the identification of methanophenazine as an electron carrier in the membrane of *Ms. mazei* it became evident that the key enzymes of the membrane-bound electron transfer systems were able to interact with 2-hydroxy-phenazine which is a water-soluble homologue of methanophenazine (Bäumer *et al.*, 2000). To analyze the key enzymes of this system the following procedure can be followed:

2.2.1. F_{420}-nonreducing hydrogenase

Several types of nickel-iron hydrogenases were described in methanogenic archaea (Vignais *et al.*, 2001). Among them is the F_{420}-nonreducing hydrogenase, which is composed of a small electron transfer subunit and a large catalytic subunit harboring a nickel-iron center. Both subunits are located at the outer leaflet of the cytoplasmic membrane. It has been shown that the metals in the bimetallic reaction center are directly involved in H_2 cleavage to 2 e^- and 2 H^+. The small subunit contains three Fe/S clusters and is responsible for electron transport from the

catalytic center to the third subunit, which is a b-type cytochrome. This subunit forms the membrane anchor and transfers electrons to methanophenazine (Deppenmeier, 2002a).

Procedure: The membrane-bound hydrogenase is assayed in 1.5-ml glass cuvettes gassed with H_2 and closed with rubber stoppers. After gassing for 5 min the cuvette is filled with 1 ml anaerobic 40 mM K-phosphate buffer, pH 7.0, containing 5 mM dithiothreitol and 1 µg mL^{-1} resazurin, and 10-15 µg of the membrane fraction is added. The reaction is started by addition of 10 mM methyl viologen (ε_{604}= 13.6 cm^{-1} x mM^{-1}). The H_2-dependent 2-hydroxyphenazine reduction is followed photometrically at 425 nm (ε = 4.5 mM^{-1} x cm^{-1}) by adding 4 µl of 2-hydroxy-phenazine from a 20 mM stock solution in ethanol. The activity should be between 1.5-2 U / mg membrane protein.

2.2.2. Heterodisulfide reductase

The heterodisulfide reductase catalyzes the reduction of CoM-S-S-CoB, which is the final step in the anaerobic respiratory chain of *Ms. mazei* and other *Methanosarcina* strains (Heiden *et al.*, 1994; Künkel *et al.*, 1997; Hedderich *et al.*, 2005). *In vivo* electrons for CoM-S-S-CoB reduction derive from the reduced form of methanophenazine (Fig. 1, 2). *In vitro* the enzyme accepts electrons from the artificial electron donors benzyl viologen and 2-hydroxyphenazine (Fig. 2).

Procedure: The benzyl viologen-dependent heterodisulfide reductase activity is determined at room temperature in 1 mL 40 mM K-phosphate buffer, pH 7.0, preflushed with N_2. After addition of 2 µL 1 M benzyl viologen, 3 µL 50 mM Na-dithionite and 1-5 µL of the membrane preparation, the reaction is started by addition of CoB-S-S-CoM to a final concentration of 180 µM and followed at 575 nm ($\varepsilon_{\text{benzyl viologen}}$ = 8.9 mM^{-1} x cm^{-1}). Typical activities range between 1-2 U / mg membrane protein.

2-hydroxyphenazine (0.25 mM final concentration) is reduced in 25 mM MOPS buffer (pH 7.0) containing 2 mM dithioerythritol, 1 µg mL^{-1} resazurin containing platinum(VI)-oxide (2 mg/ 40 mL) under a hydrogen atmosphere overnight. After reduction is completed the catalyst is removed by centrifugation in an anaerobic chamber and the resulting solution is kept under nitrogen. For the determination of membrane-bound heterodisulfide reductase activity, 25 mM MOPS buffer (pH 7.0) containing 2 mM dithioerythritol, 1 µg mL^{-1} resazurin and the reduced 2-hydroxy-phenazine solution are mixed in a 1:1 ratio (final volume 1 ml). After washed membranes are added, the reaction is started by addition of CoB-S-S-CoM to a final concentration of 180 µM and followed at 425 nm ($\varepsilon_{\text{2-hydroxyphenazine}}$ = 4.5 mM^{-1} x cm^{-1}). Typically, the activity is in the range of 2 U / mg membrane protein.

2.2.3. H_2:heterodisulfide oxidoreductase (coupled reaction of F_{420}-nonreducing hydrogenase and heterodisulfide reductase)

The entire electron transport within the H_2:heterodisulfide oxidoreductase system (Fig. 2) cannot be followed by photometric methods. However, the end product of the reaction, the thiols HS-CoM and HS-CoB (Fig. 2), can be determined using the so-called Ellman's test in which 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, also known as Ellman's reagent) reacts with mercaptans, like HS-CoM and HS-CoB, under alkaline conditions (Ellman, 1958; Zahler and Cleland, 1968). In the course of the reaction the yellow *p*-nitrobenzenethiol anion is released, which has a molar extinction coefficient of 13.6 mM^{-1} x cm^{-1} at 412 nm.

Procedure:

1. Rubber-stoppered glass vials are flushed with N_2 and 250 µL K-phosphate buffer (40 mM, pH 7.0, 1 µg mL^{-1} resazurin, flushed with N_2) is added. The solution is reduced until it just becomes colorless (using e.g. titanium(III)citrate). Approximately 150 µg washed *Ms. mazei* membranes are added, and the whole vial is flushed with 100 % H_2.
2. The reaction is started by addition of 400 µM heterodisulfide and the first sample is directly taken: a 20 µL sample of the reaction mixture is mixed with 950 µL Tris-HCl (150 mM, pH 8.1) and 100 µL DTNB (5 mM in 50 mM Na-acetate, pH 5.0) and the absorption at 412 nm is immediately measured.
3. Samples are continuously retrieved until the heterodisulfide is completely reduced ($\Delta A \sim 0.2$). Typically about 300 nmol thiols are produced per min per mg membrane protein.

Note: A buffer without mercaptan-containing reducing agent is needed for the assay, thus the buffer must not be reduced with dithioerythritol or dithiothreitol. Instead, titanium(III)citrate (Zehnder and Wuhrmann, 1976) or other mercaptan-free reducing agent can be used. The titanium(III)citrate solution is prepared as follows: 15 mmol potassium citrate is solvated in 48.75 mL H_2O and gassed with N_2 for 10 min. Carefully, 9.38 mL of a 12 % Ti(III)chloride solution (in 12 % HCl) are added under nitrogen

gassing. The pH is adjusted to 7 with a saturated K_2CO_3 solution.

2.2.4. Preparation of the heterodisulfide (CoM-S-S-CoB)

The heterodisulfide CoM-S-S-CoB is not commercially available and needs to be synthesized by condensation of HS-CoB and HS-CoM. HS-CoB (N-7-mercaptoheptanoyl-L-threonine phosphate) has to be synthesized *de novo* (Ellermann *et al.*, 1988), whereas HS-CoM (2-mercaptoethanesulfonate) is available from various suppliers.

A. Synthesis of N-hydroxysuccinimidester according to (Noll et al., 1987)

1. In a 500 mL Erlenmeyer flask 200 mL pure ethanol, 17.8 g 7-bromo-heptanoic acid (85.3 mmol) and 32.4 g thiourea (426.5 mmol) are added. The mixture is refluxed at 78° C for 16 h.
2. The solution is cooled to room temperature and alkalized with 50 mL NaOH (60 % w/v). The mixture is again refluxed at 78° C for 16 h. After cooling to room temperature, the solution is acidified with HCl to a pH < 1.
3. The solution is extracted three times with 50 mL chloroform (total 150 mL). The chloroform phases are combined and extracted with 1 M aqueous Na_2CO_3. The aqueous phase is acidified with 50 mL HCl (37 %) and extracted with chloroform (2 x 100 mL). An aqueous solution of iodine (10 % w/v)/potassium iodide (20 % w/v) is added until a light brown colour appears.
4. The aqueous phase is removed, and the chloroform phase is washed with water three times. Then the solution is dried with anhydrous $MgSO_4$ and concentrated under reduced pressure.
5. The resulting reddish-brown substance is solvated in benzene/pentane (1:1, v/v) under gentle warming, and the substance is allowed to crystallize at 4° C. The resulting crystals are filtered and re-crystallized using the same solvents. Light yellow dithiodiheptanoic acid crystals form. The yield should be in the range of 4-5 g.
6. The disulfide product is activated by synthesis of its N-hydroxysuccinimide ester using dicyclohexylcarbodiimide. 2 g dithiodiheptanoic acid, 1.5 g N-hydroxysuccinimide and 2.6 g dicyclohexylcarbodiimide (DCCD) are solvated in 60 mL anhydrous 1,4-dioxane and incubated for 20 h at room temperature under stirring. The suspension is filtered, the solid matter (dicyclohexylurea) is discarded and the filtrate is dried under vacuum to give a white solid. The white solid is recrystallized twice using 2-propanol to yield approximately 2 g N-hydroxysuccinimidester.

B. Synthesis of N-7-mercaptoheptanoyl-L-threonine phosphate (HS-CoB) according to (Kobelt et al., 1987)

1. 546 mg L-threonine phosphate (2.74 mmol) are solvated in 5 mL H_2O including 760 µL triethylamine. The mixture is stirred and 672 mg (1.30 mmol) N-hydroxysuccinimidester is solvated in 40 mL tetrahydrofuran and 14 mL acetonitrile are added. The mixture is stirred for 36 h under argon and then concentrated using a rotary evaporator. The volume is brought to approximately 40 mL with H_2O. The pH is adjusted to 6.7 with 1 M NaOH or HCl and the solution gassed with N_2. The disulfide compounds are reduced by the addition of $NaBH_4$. The pH is titrated to pH 0 with 1 M HCl.
2. For a hydrophobic interaction chromatography, an XAD-2 column (2.5 cm x 8 cm) is prepared. The column is washed with water and equilibrated with 1 M HCl before the reaction mixture is applied. The column is then washed with 100 mL HCl (1 M) and 140 mL H_2O.
3. N-7-mercaptoheptanoyl-L-threonine phosphate (HS-CoB) is eluted using a linear gradient of 0-50% methanol/H_2O (100 ml). The thiol-containing elution fractions are pooled and concentrated under vacuum. To analyze the thiol content, the following test is performed: Mix 950 µL Tris-HCl (150 mM pH 8.1), 50 – 100 µL sample and a few grains $NaBH_4$. Let the mixture stand open for 10 min, add 100 µL acetone and 100 µL DTNB solution (5 mM in 50 mM Na-acetate pH 5.0), measure at 412 nm against a control without sample (Ellmann, 1957; Zahler and Cleland 1967).

C. Synthesis of the heterodisulfide of N-7-mercaptoheptanoyl-L-threonine phosphate (HS-CoB) and 2-mercaptoethanesulfonate (HS-CoM) according to (Ellermann et al., 1988)

1. The pH of the N-7-mercaptoheptanoyl-L-threonine phosphate solution (coenzyme B solution) is adjusted to 6.7 with 1 M NaOH/HCl and reduced with a few grains $NaBH_4$ under a N_2 atmosphere. The approximate content of HS-CoB is calculated using the thiol test as described above (MW (HS-CoB) = 395 g mol^{-1}) and a 5-fold access of HS-CoM is added. The pH is adjusted to 9 (1 M NaOH) and the reaction mixture is gassed with air for 16 h at room temperature to allow the formation of the mixed disulfide.
2. After adjustment to pH 0 (1 M HCl) the preparation is applied to a XAD-2 column, equilibrated as described above. The column is washed with 150 mL 1M HCl, 250 mL H_2O

(elution of HS-CoM and CoM-S-S-CoM). The heterodisulfide CoM-S-S-CoB is eluted with 250 mL 20 % methanol, and subsequently concentrated under vacuum, frozen and lyophilized.

3. The content of biologically active heterodisulfide is determined with a biological test: 700 µL K-phosphate buffer (pH 7.0, 5 mM dithioerythritol, 1 µg mL^{-1} resazurin), 5 mM methyl viologen (ε_{604} = 13.6 mM^{-1} x cm^{-1}) and 50 – 100 µg *Ms. mazei* cytoplasmic membrane preparation (see above) are mixed in an anaerobic rubber-stoppered cuvette. The reaction mixture is titrated with Na-dithionite till an absorption of about 2 at 604 nm is reached. The test is started with a small amount of the synthesized heterodisulfide (solvated in H_2O). From the change of absorbance at 604 nm the approximate content can be calculated. The typical yield is 15-50 µmol heterodisulfide.

2.2.5. Preparation of 2-hydroxyphenazine according to (Ott, 1959)

It was found that the condensation of *p*-benzoquinone with *o*-phenylenediamine in ethanolic solution leads directly to 2-hydroxyphenazine.

1. 500 mg (4.6 mmol, 1.0 equiv.) of 1,4-benzoquinone is solvated in 5 ml of dry ethanol by slight warming.
2. Cool to room temperature, add 500 mg (4.6 mmol, 1.0 equiv.) of *o*-phenylenediamine and incubate for 1 h at ambient temperature.
3. Heat to 75°C and incubate for another 30 min.
4. Cool to room temperature and add 1 mL of H_2O; incubate for 8 h at 4°C.
5. Filter the cooled solution and solvate the brownish-black crystals in 0.5 M NaOH.
6. Filter again and acidify the filtrate with concentrated acetic acid. A brownish compound will precipitate.
7. Repeat the filtration and dry the precipitation under vacuum.
8. Purify the compound by silica gel chromatography (silica gel 60, mesh 0.05-0.20 mm, Merck) with a linear gradient of the solvent system diethylether/ petroleum ether (3:1, v/v)/diethylether.

2-hydroxyphenazine is a slight yellow solid. The yield is about 10%.

The natural electron carrier methanophenazine can also be chemically synthesized (Beifuss and Tietze 2005; Beifuss *et al.* 2000) It is a multi-step chemical synthesis and is not described here.

2.3. Analysis of the $F_{420}H_2$-dependent electron transport chain

During methanogenesis from methylated C_1-compounds in the absence of H_2, one quarter of the methyl groups is oxidized to CO_2 and reducing equivalents are in part transferred to the central electron carrier F_{420}. In the reductive branch of the pathway, three out of four methyl groups are transferred to coenzyme M. Methyl-S-CoM is reductively cleaved by methyl-S-CoM reductase, forming methane and CoB-S-S-CoM (Deppenmeier, 2002a). Thus, $F_{420}H_2$ and CoB-S-S-CoM are generated as electron donor and acceptor for the electron transport chain of the membrane-bound $F_{420}H_2$:heterodisulfide oxidoreductase system. Reduced F_{420} is oxidized by $F_{420}H_2$ dehydrogenase and CoB-S-S-CoM is reduced by heterodisulfide reductase, respectively (Fig. 2). These two enzymes are interconnected by the membrane-soluble electron carrier methanophenazine (Deppenmeier, 2002a).

2.3.1. Photometric analysis

$F_{420}H_2$ dehydrogenase activity is determined at room temperature in glass cuvettes under N_2**.** The 700 µL anaerobic assay mixture routinely contains: 40 mM K-phosphate buffer, pH 7.0, 20 mM $MgSO_4$, 0.5 M sucrose, 1 µg mL^{-1} resazurin, 10 mM dithioerythritol and 16 µM $F_{420}H_2$. The $F_{420}H_2$ dehydrogenase reaction is started with 0.5 mM metronidazole and 0.3 mM methyl viologen. Metronidazole re-oxidizes reduced methyl viologen, which is necessary because the concentration of reduced methyl viologen has to be kept low to prevent the spectroscopic interference with F_{420}. The oxidation of $F_{420}H_2$ is followed at 420 nm (ε = 40 mM^{-1} x cm^{-1}). The activity is about 50 mU / mg membrane protein.

$F_{420}H_2$:heterodisulfide oxidoreductase activity is determined similarly to the $F_{420}H_2$ dehydrogenase activity except metronidazole and methyl viologen are replaced by the intrinsic electron acceptor heterodisulfide to a final concentration of 20 µM. Typical activities range between 50 and 200 mU / mg membrane protein. As the $F_{420}H_2$ dehydrogenase interacts with the membrane-soluble methanophenazine pool, the terminal electron acceptor heterodisulfide can be replaced by the intermediary electron acceptor 2-hydroxyphenazine, which is used at a final concentration of 50 µM. An activity of about 200 mU / mg membrane protein is to be expected.

2.3.2 Purification and reduction of coenzyme F_{420}

The ultracentrifugation supernatants from membrane or vesicle preparations contain all cytoplasmic compounds and can be used for the preparation of F_{420}. Alternatively, cells of *Ms. barkeri* or *Ms. mazei* can be used. The preparations are suspended under N_2 in 40 mM NH_4-acetate buffer, pH 4.8, containing 10 mM 2-mercaptoethanol. The preparations are boiled in a water bath for 30 min under an atmosphere of argon, sedimented by centrifugation at 25,000 x *g* for 30 min and resuspended in the same buffer. Boiling and centrifugation is repeated, and the supernatants of both extractions are combined and applied to a DEAE-Sephadex A25 anion exchange column, which has been equilibrated with a 40 mM NH_4-acetate buffer, pH 4.8. A linear 1L gradient (0 to 1 M NH_4Cl in NH_4-acetate buffer) is applied and fractions containing F_{420} are detected by their absorption at 420 nm. Pooled fractions are diluted 10-fold with 50 mM Tris-HCl buffer, pH 7.5, and loaded onto a second DEAE-Sephadex column (8 cm x 4 cm). The cofactor is eluted with a 0 to 1 M NaCl gradient in the same buffer. The salt content of the green fluorescent solution of F_{420} is reduced by diluting with 50 mM Tris-HCl buffer, pH 7.5, and the preparation is placed on a QAE-Sephadex column (2.6 cm by 7 cm) that has been equilibrated with the same buffer. A 0.5 L gradient of 0 to 2 M NaCl is passed through the column. Finally, the F_{420}-containing fractions are adjusted to pH 1 with 6 M HCl and applied to a XAD-4 column (2.6 cm x 5 cm). The preparation is desalted by applying 100 mL 1 M HCl and F_{420} is eluted with a linear methanol gradient (0 – 100 %). The F_{420} containing fractions are combined, neutralized with NaOH and concentrated by rotary evaporation.

Coenzyme F_{420} is reduced under a flow of N_2 by adding $NaBH_4$, and incubating at room temperature until the solution turns colourless. Excess $NaBH_4$ is removed by adding anaerobic HCl to a final pH of 0. Anaerobic NaOH is used to readjust to pH 7.0. As the volume of this reaction is rather low, care must be taken not to lose significant amounts of material due to monitoring pH. Ideally, only 1 µL of the solution is applied to an appropriate pH test strip. After readjustment of the pH, the vial is sealed with a rubber stopper, and is used the same day. The $F_{420}H_2$ solution is not suitable for storage and will slowly oxidize.

2.4. Analysis of the ferredoxin-dependent electron transport chain

The major part of methane produced by the anaerobic breakdown of organic matter derives from the methyl group of acetate (Ferry and Lessner, 2008). The process is referred to as the aceticlastic pathway of methanogenesis. Only species of the genera *Methanosarcina* and *Methanosaeta* are known to produce CH_4 and CO_2 from acetate. *Methanosarcina* species activate acetate by conversion to acetyl-coenzyme A (CoA). The key enzyme in the aceticlastic pathway is the CO-dehydrogenase/acetyl-CoA synthase which is responsible for the cleavage of the C-C and C-S bonds in acetyl-CoA (Gong *et al.*, 2008; Ragsdale, 2008; Gencic *et al.*, 2010). This enzyme also catalyzes the oxidation of CO to CO_2 and the electrons are used for ferredoxin reduction (Clements *et al.*, 1994). The methyl group of acetyl-CoA is transferred to H_4SPT, forming methyl-H_4SPT (Fischer and Thauer, 1989), which is converted to methane by the Na^+-translocating methyl-H_4MPT:HS-CoM methyltransferase (Gottschalk and Thauer, 2001) (see chapter xx of this volume) and the methyl-S-CoM reductase as described above (Ebner *et al.*, 2010). Again, the heterodisulfide CoM-S-S-CoB is formed and is reduced by a membrane-bound electron transport system (Fig. 2). In *Ms. mazei* and *Ms. barkeri* the aceticlastic respiratory chain comprises the three transmembrane proteins Ech hydrogenase (Meuer *et al.*, 1999; Meuer *et al.*, 2002; Hedderich and Forzi, 2005), F_{420}-nonreducing hydrogenase (Ide *et al.*, 1999) and heterodisulfide reductase (Bäumer *et al.*, 2000) (Fig. 2). The oxidation of reduced ferredoxin is catalyzed by Ech hydrogenase resulting in the release of molecular hydrogen (Meuer *et al.*, 1999) that is then re-oxidized by the F_{420}-nonreducing hydrogenase and the electrons are channelled via methanophenazine to the heterodisulfide reductase (Welte *et al.*, 2010a; Welte *et al.*, 2010b). The entire electron transport system is referred to as ferredoxin:heterodisulfide oxidoreductase. In addition to aceticlastic methanogenesis, reduced ferredoxin is produced in the oxidative branch of methylotrophic methanogenesis. As previously described in the section “Analysis of the $F_{420}H_2$-dependent electron transport chain”, ¾ of the methyl groups are used to generate the heterodisulfide with methane as a side product. A quarter of the methyl groups are oxidized to yield reducing equivalents in the form of two molecules of $F_{420}H_2$ and two reduced ferredoxins per methyl group oxidized. The enzyme responsible for the reduction of ferredoxin is the formyl-methanofuran dehydrogenase that oxidizes a formyl group to CO_2 (Bartoschek *et al.*, 2000).

Ferredoxin-mediated electron transport can be analyzed using washed cytoplasmic membranes that comprise all essential proteins involved in aceticlastic energy conservation (Welte *et al.*, 2010a). However, these membranes do not contain a system to generate reduced ferredoxin. Therefore, a system of *Moorella thermoacetica* CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) and *Clostridium pasteurianum* ferredoxin (Fd_{Cl}) is used. In this small electron transport chain, CO is oxidized by CODH/ACS, which transfers electrons to ferredoxin. Clostridial ferredoxin can specifically donate electrons to Ech hydrogenase and initiate electron transport in the ferredoxin:heterodisulfide oxidoreductase system (Welte *et al.*, 2010b).

2.4.1. Analysis of the Fd:heterodisulfide oxidoreductase (coupled reaction of Ech hydrogenase, F_{420}-nonreducing hydrogenase and heterodisulfide reductase)

The Fd:heterodisulfide oxidoreductase system can be analyzed using the Ellman's test as described above under "H_2:heterodisulfide oxidoreductase".

1. Rubber-stoppered glass vials are flushed with N_2. 250 µL K-phosphate buffer (40 mM, pH 7.0, 1 µg mL^{-1} resazurin, flushed with N_2) is added and reduced until the solution just becomes colorless (using e.g. titanium(III)citrate). Approximately 150 µg washed *Ms. mazei* membranes are added and the whole vial is flushed with 5 % CO / 95 % N_2. 30 µg clostridial ferredoxin and 70 µg CODH/ACS are added.
2. The reaction is started by addition of 400 µM heterodisulfide and the first sample is directly taken: a 20 µL sample of the reaction mixture is mixed with 950 µL Tris-HCl (150 mM, pH 8.1) and 100 µL DTNB (5 mM in 50 mM Na-acetate, pH 5.0) and the absorption at 412 nm is immediately measured.
3. Samples are taken until the heterodisulfide is completely reduced (ΔA ~ 0.2). Normally 70 nmol thiols are produced per min per mg membrane protein.

2.4.2. Ech hydrogenase: Quantification of molecular hydrogen

The activity of Ech hydrogenase can be determined independently of the complete Fd:heterodisulfide oxidoreductase. Ech hydrogenase catalyzes H_2 formation in the process of ferredoxin oxidation (Meuer *et al.*, 1999). So when washed cytoplasmic membranes of *Ms. mazei* are provided with reduced ferredoxin, they will produce molecular hydrogen. To measure H_2 production rates, rubber stoppered glass vials are filled with 500 µL K-phosphate buffer (40 mM, pH 7.0, 1 µg mL^{-1} resazurin, 5 mM dithioerythritol), 5 % CO / 95 % N_2 in the 1.5 mL headspace, 500-700 µg washed cytoplasmic membrane preparation, 33.5 µg Fd_{Cl} and 20 µg CODH/ACS. The vials are shaken at 37° C, and 10 µL of the headspace are injected into a gas chromatograph connected to a thermal conductivity detector with argon as carrier gas at various time points. The resulting peak areas can be compared with standard curves with defined hydrogen content. Expected activities range between 30-60 nmol H_2 / mg membrane protein.

2.4.3. Preparation of ferredoxin

Clostridial ferredoxin (Fd_{Cl}) can easily be prepared following a protocol developed by Mortenson (Mortenson, 1964). The steps can be performed aerobically. However, if Fd_{Cl} is stored for a prolonged time under the influence of oxygen the iron-sulfur centers will decompose and the protein will lose its colour and function.

For the preparation of Fd_{Cl}, 2-4 L *Clostridium pasteurianum* DSM 525 culture are pelleted and resuspended in water. The cells are lysed by lyzozyme treatment and sonication (to improve lysis, the cell suspension can be frozen or lyophilized prior to sonication). The volume of the crude extract is brought to 150 – 200 mL with ice-cold water. An equal volume of ice-cold acetone is added and the solution is stirred for 15 min in an ice bath. The solution is centrifuged for 15 min at 7000 x *g* and 4 ℃, and the supernatant is applied onto an anion exchange column (DEAE cellulose, 2.5 x 8 cm, equilibrated with 50 % (v/v) acetone). Ferredoxin directly becomes visible on top of the chromatography column as a dark brown ring. The column is washed with 200 mM Tris-HCl pH 8.0 until the bright yellow flavins no longer elute from the column (~ 100 mL). Fd_{Cl} is eluted with 500 mM Tris-HCl pH 8.0. The brown fractions are concentrated via ultrafiltration, and stored aerobically at – 70° C in aliquots.

2.4.4. Preparation of CO dehydrogenase/acetyl-CoA synthase

CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) can be purified from *Moorella thermoacetica* ATCC 39073 as described by Ragsdale *et al.*, (1983), with modifications as specified (Welte *et al.*, 2010a). All steps should be carried out anaerobically.

1. *Moorella thermoacetica* cells are lysed (suspended in 50 mM Tris-HCl, pH 7.5, 2 mM Na-dithionite) by French press treatment.
2. The lysate is applied to a DEAE cellulose column and eluted with a step gradient of NaCl from 0.1 M to 0.5 M, CODH/ACS elutes at 0.3 M NaCl. To screen for the CODH/ACS containing

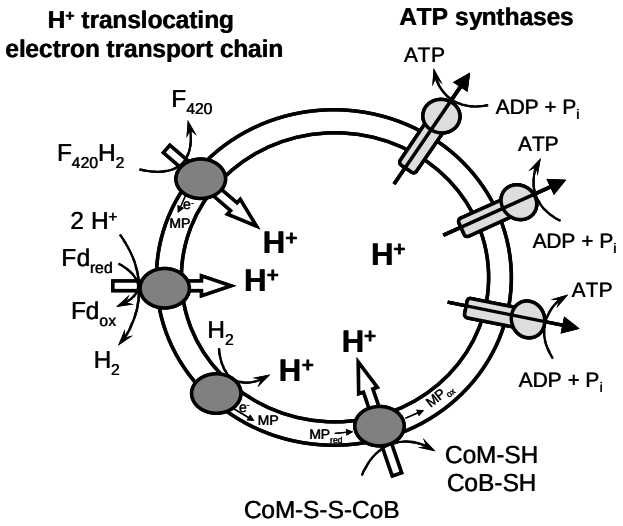

Figure 3: Scheme of inside-out vesicles.

fractions, the following enzymatic test is used: 700 µL K-phosphate buffer (40 mM, pH 7.0, 1 µg mL^{-1} resazurin, 5 mM dithioerythritol), 5 mM methyl viologen, 5 % CO in the headspace. The reaction can be monitored at 600 nm at room temperature.

3. The enzyme containing fractions are applied to a Q-sepharose anion exchange column (elution at approximately 0.4 M NaCl) and a phenyl-sepharose hydrophobic interaction column (elution at approximately 0.2 M $(NH_4)_2SO_4$).

4. The fractions from the phenyl-sepharose column that contain CODH/ACS are concentrated and the buffer is exchanged into 50 mM Tris-HCl (pH 7.6) by ultrafiltration.

3. Measurement of proton translocation

As described above, the membrane-bound electron transfer of *Ms. mazei* has been analyzed in detail. To elucidate the coupling between electron transport and ion translocation, a subcellular system of this organism was developed that consisted of washed inverted membrane vesicles free from cytoplasmic components (Mayer *et al.*, 1987; Deppenmeier *et al.*, 1988). The advantage is that enzymes located at the inner leaflet of the membrane are accessible to the highly charged substrates F_{420} and CoM-S-S-CoB because of the inverted orientation of these vesicles. Using these vesicle preparations, it has been shown that electron transport as catalyzed by the Ech hydrogenase, the $F_{420}H_2$: heterodisulfide oxidoreductase system and the H_2:heterodisulfide oxidoreductase system is accompanied by proton transfer across the cytoplasmic membrane into the lumen of the inverted vesicles (Deppenmeier, 2004; Welte *et al.*, 2010b).

3.1. Preparation of washed inverted vesicles

In contrast to other *Methanosarcina* species, *Ms. mazei* lacks a heteropolysaccharide layer and only possesses a proteinaceous cell wall (Jussofie *et al.*, 1986). By treatment with proteases the protein layer can be digested resulting in the formation of protoplasts. Gentle French pressure treatment and centrifugation results in the formation of cell-free lysate containing membrane vesicles with 90 % inside-out orientation (Fig. 3). Inside-out oriented membrane vesicles have the great advantage that the active sites of most energy-transducing enzymes face the outside and are accessible for extraneously added electron donors or acceptors (e.g. $F_{420}H_2$, Fd_{red}, heterodisulfide). Thus, inside-out oriented membrane vesicles offer a variety of possibilities to investigate membrane-bound energy transduction mechanisms. To produce inverted membrane vesicles, the following procedure can be used:

1. Grow at least 4 L of *Ms. mazei* culture. All steps should be performed anaerobically. Harvest the cells in the late exponential growth phase (OD_{600} 0.8 – 1) and resuspend the cells in 20 – 50 mL vesicle buffer (20 mM K-phosphate buffer, pH 7.0, 20 mM $MgSO_4$, 500 mM sucrose, 10 mM dithioerythritol, 1 µg mL^{-1} resazurin). Gently homogenize the cells.

2. For protoplast formation, add 2 mg pronase per L culture volume. Incubate at 37° C until protoplasts appear (this can be visualized by light microscopy; the cells become perfectly globe-shaped), do not exceed 30 min. Stop the reaction by addition of ~50 µL PMSF (100 mM in isopropanol), and directly put the protoplast vial on ice. Always handle protoplasts (and vesicles) very gently!

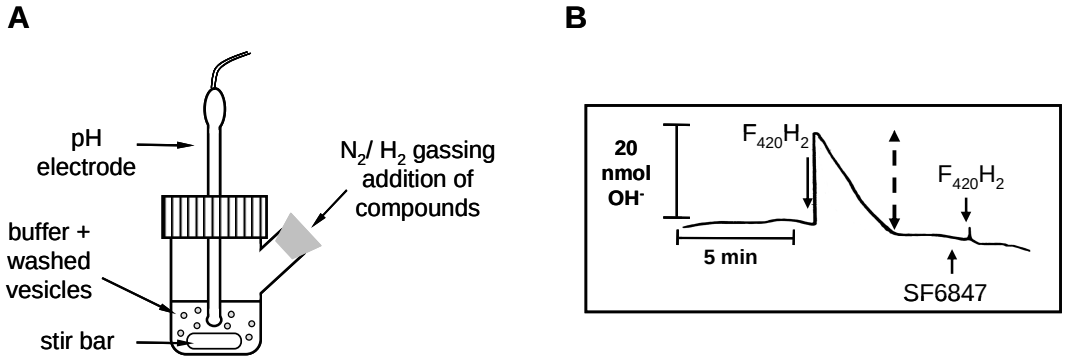

Figure 4: Analysis of proton translocation. A) Device for continuous determination of the pH value, B) flow chart of proton translocation, black arrows indicate the addition of $F_{420}H_2$ or the protonophor SF6847, the broken arrow shows the extent of alkalinization.

3. Centrifuge the protoplast suspension at 12,000 x *g* for 10 min at 4° C. Resuspend the pellet in 30 mL vesicle buffer containing DNase. Apply the preparation to a French press cell within an anaerobic chamber and connect the outlet with a N_2-gassed and rubber-stoppered serum bottle by using a short tube ending with a needle which is injected into the serum flask. Take the cell out and French press the washed protoplast suspension at 600 psi. The preparation is released into the anaerobic serum bottle (caution: omit overpressure by inserting a second needle!). The result is a crude inverted vesicle preparation with 90 % inside-out orientation, which is then transferred to appropriate centrifugation bottles within the anaerobic chamber. Spin the crude vesicle preparation at 1,300 x *g* for 10 min at 4° C. The supernatant contains the crude vesicles and should be turbid. Note: sometimes the vesicles form a slight brown pellet and should be included in the ultracentrifugation (see below)!

4. Ultracentrifuge the crude vesicle preparation at 150,000 x *g* for 60 – 90 min at 4° C. The brown pellet consists of the inverted membrane vesicles. Often, a black pellet underneath is observed which contains sulfidic contaminants and should be discarded. Carefully remove the cytoplasmic supernatant (be careful not to remove the pellet: it is easily disturbed) and resuspend the pellet in vesicle buffer. Transfer the vesicle suspension to a new ultracentrifugation vial and re-centrifuge (30 – 60 min is sufficient) until the supernatant becomes colourless. Note: care must be taken in the initial centrifugation step, as membrane vesicles will not pellet if they are too concentrated. In this case the vesicles must be diluted.

5. Finally, resuspend the washed inverted membrane vesicle preparation in 500 – 2000 µL vesicle buffer (final protein concentration ~ 25 mg mL^{-1}), aliquot and freeze them anaerobically at -70° C under an H_2 atmosphere.

3.2. Experimental determinations of the $H^+/2e^-$ ratio

In most organisms electron flow through the respiratory chain is directly coupled to the formation of an electrochemical proton gradient (□p) that is formed by protein-catalysed proton transfer across biological phospholipid bilayers. This process is also performed by the methanogenic archaeon *Ms. mazei* and is coupled by the electron transfer from the electron input modules F_{420}-nonreducing hydrogenase and $F_{420}H_2$ dehydrogenase to the heterodisulfide reductase (Deppenmeier *et al.*, 1990; Deppenmeier *et al.*, 1992). Furthermore, it was shown that the Ech hydrogenase also contributes to the electrochemical proton gradient (Welte *et al.*, 2010a; Welte *et al.*, 2010b). One important aspect of this phenomenon is the stoichiometric relationship between proton translocation and electron transport, which is expressed by the $H^+/2e^-$ ratio.

Procedure: Proton translocation can be followed in a glass vessel (5.5 ml total volume) filled with 2.5 ml 40 mM potassium thiocyanate, 0.5 M sucrose, 0.1 mg/ml resazurin and 10 mM dithioerythritol (Fig. 4A). The thiocyanate ion is membrane permeable and prevents the formation of a membrane potential to ensure electroneutral movement of protons. A sensitive pH electrode (e.g. model 8103 Ross, Orion research, Küsnacht, Switzerland) is inserted into the vessel from the top through a rubber stopper. Connect the electrode with a pH meter (e.g. Orion model EA 920) and a chart recorder. The vessel is subsequently gassed for 10 min with N_2 by means of two needles inserted from the side through a rubber stopper (Fig. 4A). In case the

solution is not totally colourless, add up to 1 µL titanium(III) citrate for complete reduction. Then 100 µL washed vesicles (0.35-0.5 mg protein/assay) are added, resulting in a final K-phosphate concentration of 1.5 mM in the reaction mixture. The preparation is continuously stirred and the pH is adjusted to 6.8-6.9. Additions are made with a microliter syringe from the side arm.

As mentioned above, proton translocation is due to electron transfer from an electron donor (H_2, $F_{420}H_2$, reduced 2-hydroxyphenazine) to an acceptor (2-hydroxyphenazine or CoM-S-S-CoB). In principle one substrate is added in excess, whereas the second substrate is injected in low amounts ("pulse injection"; usually 2-10 nmol) that allows only a short period of electron transfer events. The pulse injection will lead to alkalinization of the medium as is apparent from the recorded time course of this process (Fig. 4B). A short period of alkalinization is then followed by a longer period of acidification until a stable baseline (pH) is reached again. The alkalinization is due to proton movement from the medium into the lumen of the inverted vesicles energized by the electron transfer reactions. The extent of alkalinization is dependent on the amount of the pulsed substrate. Changes of the pH value are converted into proton equivalents by double titration with standard solutions of HCl and KOH (Fig. 4B). The amount of H^+ translocated is calculated from the difference between the maximum of alkalinization and the final baseline after reacidification (Fig. 4B). The ratio of translocated protons and transferred electron can be calculated since the amount of pulsed substrate is known.

Reaction conditions:

- H_2: heterodisulfide oxidoreductase: Use an H_2 atmosphere and pulse with CoM-S-S-CoB (2-10 nmol in oxygen free H_2O)
- F_{420}-nonreducing hydrogenase: Use an H_2 atmosphere and pulse with hydroxyphenazine (2-10 nmol from a 1 mM stock solution solvated in anoxic ethanol)
- Heterodisulfide reductase: N_2 atmosphere in the reaction vessel; add 250 nmol CoM-S-S-CoB and pulse with reduced hydroxyphenazine (2-10 nmol from a 1 mM stock solution solvated in anoxic ethanol)
- $F_{420}H_2$ dehydrogenase: N_2 atmosphere in the reaction vessel; add 250 nmol hydroxyphenazine and pulse with $F_{420}H_2$ (2-10 nmol)
- $F_{420}H_2$: heterodisulfide oxidoreductase: N_2 atmosphere in the reaction vessel; add 250 nmnol CoM-S-S-CoB and pulse with reduced F_{420} (2-10 nmol)

In our studies, the reversible alkalinization was used to calculate from more than 60 experiments an average of 1.9 protons translocated per $F_{420}H_2$ oxidized in the $F_{420}H_2$: heterodisulfide oxidoreductase assay. The observed alkalinization was specifically coupled with the $F_{420}H_2$-dependent CoM-S-S-CoB reduction. Alkalinization was not observed if $F_{420}H_2$ or CoM-S-S-CoB was omitted or if $F_{420}H_2$ was replaced by F_{420}. Similar values were obtained when the H_2:heterodisulfide oxidoreductase system was analysed. Keeping in mind that about 50 % of the membrane structures present in the vesicle preparations catalyze electron transport but are unable to establish a proton gradient (Deppenmeier *et al.*, 1996), the $H^+/2e^-$ values of the overall reaction increases to 3.8, which is in agreement with values found for whole cell preparations of *Ms. barkeri* (Blaut *et al.*, 1987).

The aforementioned washed inverted vesicles were found to couple electron transfer processes with the transfer of about four protons across the cytoplasmic membrane. Furthermore, it was shown that 2-hydroxyphenazine is reduced by molecular hydrogen, as catalyzed by the F_{420}-nonreducing hydrogenase, and the membrane-bound heterodisulfide reductase was able to use reduced 2-hydroxyphenazine as the electron donor for the reduction of CoM-S-S-CoB. In addition, the $F_{420}H_2$-dehydrogenase could use hydroxyphenazine as electron acceptor (Ide *et al.*, 1999; Bäumer *et al.*, 2000). All reactions allowed the translocation of protons with a stoichiometry of about $2H^+/2e^-$. Hence, there are two proton-translocating segments present in the $F_{420}H_2$:CoM-S-S-CoB and the H_2:CoM-S-S-CoB oxidoreductase system, respectively. The first one involves the electron donating enzymes (F_{420}-nonreducing hydrogenase or $F_{420}H_2$ dehydrogenase) and the second one the heterodisulfide reductase (Fig. 2). Control experiments showed that the formation of a proton gradient was not possible in the presence of a protonophor, such as SF6847 (10 nmol/ mg protein) (Fig. 4B). In contrast H^+ translocation was not affected by an inhibitor of the ATP synthase (e.g. DCCD; 400 µM) or in the presence of a Na^+ ionophor (e.g. ETH157; 30 µM).

4. Analysis of ATP formation in vesicle preparation

When protons are translocated into the inverted membrane vesicles by enzymes comprising the methanogenic respiratory chain, and when ADP and P_i are present, the A_1A_O ATP synthase can phosphorylate ADP in the process of proton extrusion. ATP content can be

measured using the luciferin/luciferase assay (Kimmich *et al.*, 1975). The reaction of luciferase with its substrate luciferin is strictly ATP-dependent and produces light, and the amount of ATP is directly proportional to the amount of emitted light.

Procedure: The rubber-stoppered, N_2-flushed glass reaction vial contains 500 µL vesicle buffer, 600 µM ADP, 300 µM AMP (to inhibit membrane-bound adenylate kinase), and 250-700 µg inverted membrane vesicles. Depending on the system to be observed different electron donors or acceptors need to be used. For the H_2:heterodisulfide oxidoreductase, a H_2 atmosphere and 200-500 µM heterodisulfide as electron acceptor is used. For the $F_{420}H_2$:heterodisulfide oxidoreductase, a N_2 atmosphere, 10 µL $F_{420}H_2$ (final concentration 15 µM) as electron donor and 200-500 µM heterodisulfide as electron acceptor is used. For measuring the Fd:heterodisulfide oxidoreductase, a 5 % CO / 95 % N_2 atmosphere is applied to the reaction vial. Furthermore, 70 µg CO-dehydrogenase and 30 µg Fd are added to generate Fd_{red}, and 200-500 µM heterodisulfide are added as terminal electron acceptor. If Ech hydrogenase as part of the Fd:heterodisulfide oxidoreductase is to be measured independently, the heterodisulfide can be omitted. Electrons are transferred to protons as terminal electron acceptors and form H_2.

The reaction vials are shaken in a 37° C water bath and 10 µL samples are taken at various time points with a microliter syringe. The sample is mixed with 700 µL ATP determination buffer (4 mM $MgSO_4$, 20 mM glycylglycine, pH 8.0) and 100 µL firefly lantern extract (Sigma-Aldrich, Germany). The emitted light is quantified with a luminometer at 560 nm after a lag phase of 10 s. The values are compared to a standard curve generated with 1 – 100 pmol ATP. For the H_2:heterodisulfide oxidoreductase and Ech hydrogenase an activity of about 1.5-2 nmol ATP/ mg membrane protein, and for the complete Fd:heterodisulfide oxidoreductase an activity of 3-4 nmol ATP / mg membrane protein is expected.

Acknowledgments

We thank Elisabeth Schwab for technical assistance and Paul Schweiger for critical reading of the manuscript. Many thanks also go to Gunes Bender and Steve Ragsdale, Department of Biological Chemistry, University of Michigan Medical School for providing the CODH/ACS from *Moorella thermoacetica*. This work was supported by the Deutsche Forschungsgemeinschaft (grant De488/9-1).

References

Abken, H. J., Tietze, M., Brodersen, J., Bäumer, S., Beifuss, U. and Deppenmeier, U. (1998). Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Gö1. *J. Bacteriol.* **180**(8), 2027-2032.

Bartoschek, S., Vorholt, J. A., Thauer, R. K., Geierstanger, B. H. and Griesinger, C. (2000). N-carboxymethanofuran (carbamate) formation from methanofuran and CO_2 in methanogenic archaea. Thermodynamics and kinetics of the spontaneous reaction. *Eur. J. Biochem.* **267**(11), 3130-3138.

Bäumer, S., Ide, T., Jacobi, C., Johann, A., Gottschalk, G. and Deppenmeier, U. (2000). The $F_{420}H_2$ dehydrogenase from *Methanosarcina mazei* is a redox-driven proton pump closely related to NADH dehydrogenases. *J. Biol. Chem.* **275**(24), 17968-17973.

Beifuss, U. and Tietze, M. (2000). On the total synthesis of (S)-methanophenazine and the formal synthesis of (R)-methanophenazine from a common precursor. *Tetrahedron Lett.* **41**(50), 9759-9763.

Beifuss, U., Tietze, M., Baumer, S. and Deppenmeier, U. (2000). Methanophenazine: Structure, total synthesis, and function of a new cofactor from methanogenic archaea. Angew. Chem. Int. Ed. 39 (14), 2470-

Beifuss, U. and Tietze, M. (2005). Methanophenazine and other natural biologically active phenazines. *Top. Curr. Chem.* **244**, 77-113.

Blaut, M. and Gottschalk, G. (1984). Coupling of ATP synthesis and methane formation from methanol and molecular hydrogen in *Methanosarcina barkeri*. *Eur. J. Biochem.* **141**(1), 217-222.

Blaut, M., Müller, V. and Gottschalk, G. (1987). Proton translocation coupled to methanogenesis from methanol + hydrogen in *Methanosarcina barkeri*. *FEBS Lett.* **215**(1), 53-57.

Boone, D. R., Whitman, W. B. and Rouvière, P. E. (1993). Diversity and taxonomy of methanogens. *Methanogenesis*. J. G. Ferry. New York, Chapman & Hall**:** 35-80.

Clements, A. P., Kilpatrick, L., Lu, W. P., Ragsdale, S. W. and Ferry, J. G. (1994). Characterization of the iron-sulfur clusters in ferredoxin from acetate-grown *Methanosarcina thermophila*. *J. Bacteriol.* **176**(9), 2689-2693.

Deppenmeier, U. (2002a). Redox-driven proton translocation in methanogenic archaea. *Cell. Mol. Life Sci.* **59**(9), 1513-1533.

Deppenmeier, U. (2002b). The unique biochemistry of methanogenesis. *Prog. Nucleic Acid Res. Mol. Biol.* **71**, 223-283.

Deppenmeier, U. (2004). The membrane-bound electron transport system of *Methanosarcina* species. *J. Bioenerg. Biomembr.* **36**(1), 55-64.

Deppenmeier, U., Blaut, M., Jussofie, A. and Gottschalk, G. (1988). A methyl-CoM methylreductase system from methanogenic bacterium strain Gö 1 not requiring ATP for activity. *FEBS Lett.* **241**(1-2), 60-64.

Deppenmeier, U., Blaut, M., Mahlmann, A. and Gottschalk, G. (1990). Reduced coenzyme F_{420}: heterodisulfide oxidoreductase, a proton-translocating redox system in methanogenic bacteria. *Proc. Natl. Acad. Sci. USA* **87**(23), 9449-9453.

Deppenmeier, U., Blaut, M., Schmidt, B. and Gottschalk, G. (1992). Purification and properties of a F_{420}-nonreactive, membrane-bound hydrogenase from *Methanosarcina* strain Gö1. *Arch. Microbiol.* **157**(6), 505-511.

Deppenmeier, U. and Müller, V. (2008). Life close to the thermodynamic limit: how methanogenic archaea conserve energy. *Results Probl. Cell Differ.* **45**, 123-152.

Deppenmeier, U., Müller, V. and Gottschalk, G. (1996). Pathways of energy conservation in methanogenic archaea. *Arch. Microbiol.* **165**(3), 149-163.

Ebner, S., Jaun, B., Goenrich, M., Thauer, R. K. and Harmer, J. (2010). Binding of coenzyme B induces a major conformational change in the active site of methyl-coenzyme M reductase. *J. Am. Chem. Soc.* **132**(2), 567-575.

Ellermann, J., Hedderich, R., Bocher, R. and Thauer, R. K. (1988). The final step in methane formation. Investigations with highly purified methyl-CoM reductase (component C) from *Methanobacterium thermoautotrophicum* (strain Marburg). *Eur. J. Biochem.* **172**(3), 669-677.

Ellman, G. L. (1958). A colorimetric method for determining low concentrations of mercaptans. *Arch. Biochem. Biophys.* **74**(2), 443-450.

Ferry, J. G. (1999). Enzymology of one-carbon metabolism in methanogenic pathways. *FEMS Microbiol. Rev.* **23**(1), 13-38.

Ferry, J. G. and Lessner, D. J. (2008). Methanogenesis in marine sediments. *Ann. N. Y. Acad. Sci.* **1125**, 147-157.

Fischer, R. and Thauer, R. K. (1989). Methyltetrahydromethanopterin as an intermediate in methanogenesis from acetate in *Methanosarcina barkeri*. *Arch. Microbiol.* **151**(5), 459-465.

Gencic, S., Duin, E. C. and Grahame, D. A. (2010). Tight coupling of partial reactions in the acetyl-CoA decarbonylase/synthase (ACDS) multienzyme complex from *Methanosarcina thermophila*: acetyl C-C bond fragmentation at the A cluster promoted by protein conformational changes. *J. Biol. Chem.* **285**(20), 15450-15463.

Gong, W., Hao, B., Wei, Z., Ferguson, D. J., Tallant, T., Krzycki, J. A. and Chan, M. K. (2008). Structure of the alpha(2)epsilon(2) Ni-dependent CO dehydrogenase component of the *Methanosarcina barkeri* acetyl-CoA decarbonylase/synthase complex. *Proc. Natl. Acad. Sci. USA* **105**(28), 9558-9563.

Gottschalk, G. and Thauer, R. K. (2001). The Na^+-translocating methyltransferase complex from methanogenic archaea. *Biochim. Biophys. Acta* **1505**(1), 28-36.

Hedderich, R. and Forzi, L. (2005). Energy-converting [NiFe] hydrogenases: more than just H_2 activation. *J. Mol. Microbiol. Biotechnol.* **10**(2-4), 92-104.

Hedderich, R., Hamann, N. and Bennati, M. (2005). Heterodisulfide reductase from methanogenic archaea: a new catalytic role for an iron-sulfur cluster. *Biol. Chem.* **386**(10), 961-970.

Heiden, S., Hedderich, R., Setzke, E. and Thauer, R. K. (1994). Purification of a 2-subunit cytochrome-b containing heterodisulfide reductase from methanol-grown *Methanosarcina barkeri*. *Eur. J. Biochem.* **221**(2), 855-861.

Hippe, H., Caspari, D., Fiebig, K. and Gottschalk, G. (1979). Utilization of trimethylamine and other N-methyl compounds for growth and methane formation by *Methanosarcina barkeri*. *Proc. Natl. Acad. Sci. USA* **76**(1), 494-498.

Ide, T., Bäumer, S. and Deppenmeier, U. (1999). Energy conservation by the H_2:heterodisulfide oxidoreductase from *Methanosarcina mazei* Gö1: identification of two proton-translocating segments. *J. Bacteriol.* **181**(13), 4076-4080.

Jain, N., Pathak, H., Mitra, S. and Bhatia, A. (2004). Emission of methane from rice fields - A review. *J. Sci. Ind. Res.* **63**(2), 101-115.

Jussofie, A., Mayer, F. and Gottschalk, G. (1986). Methane formation from methanol and molecular hydrogen by protoplasts of new methanogenic isolates and inhibition by dicyclohexylcarbodiimide. *Arch. Microbiol.* **146**(3), 245-249.

Kashyap, D. R., Dadhich, K. S. and Sharma, S. K. (2003). Biomethanation under psychrophilic conditions: a review. *Bioresour. Technol.* **87**(2), 147-153.

Khalil, M. A. K. and Rasmussen, R. A. (1994). Global emissions of methane during the last

several centuries. *Chemosphere* **29**(5), 833-842.

Kimmich, G. A., Randles, J. and Brand, J. S. (1975). Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. *Anal. Biochem.* **69**(1), 187-206.

Kobelt, A., Pfaltz, A., Ankelfuchs, D. and Thauer, R. K. (1987). The L-form of N-7-mercaptoheptanoyl-O-phosphothreonine is the enantiomer active as component B in methyl-CoM reduction to methane. *FEBS Lett.* **214**(2), 265-268.

Künkel, A., Vaupel, M., Heim, S., Thauer, R. K. and Hedderich, R. (1997). Heterodisulfide reductase from methanol-grown cells of *Methanosarcina barkeri* is not a flavoenzyme. *Eur. J. Biochem.* **244**(1), 226-234.

Mayer, F., Jussofie, A., Salzmann, M., Lubben, M., Rohde, M. and Gottschalk, G. (1987). Immunoelectron microscopic demonstration of ATPase on the cytoplasmic membrane of the methanogenic bacterium strain Gö1. *J. Bacteriol.* **169**(5), 2307-2309.

McInerney, M. J., Sieber, J. R. and Gunsalus, R. P. (2009). Syntrophy in anaerobic global carbon cycles. *Curr. Opin. Biotechnol.* **20**(6), 623-632.

Meuer, J., Bartoschek, S., Koch, J., Kunkel, A. and Hedderich, R. (1999). Purification and catalytic properties of Ech hydrogenase from *Methanosarcina barkeri*. *Eur. J. Biochem.* **265**(1), 325-335.

Meuer, J., Kuettner, H. C., Zhang, J. K., Hedderich, R. and Metcalf, W. W. (2002). Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. *Proc. Natl. Acad. Sci. USA* **99**(8), 5632-5637.

Mortenson, L. E. (1964). Purification and analysis of ferredoxin from *Clostridium pasteurianum*. *Biochim. Biophys. Acta* **81**(1), 71-77.

Noll, K. M., Donnelly, M. I. and Wolfe, R. S. (1987). Synthesis of 7-Mercaptoheptanoylthreonine phosphate and its activity in the methylcoenzyme-M methylreductase system. *J. Biol. Chem.* **262**(2), 513-515.

Ott, R. (1959). Untersuchungen über Chinone. 2. Mitt.: Azinbildung durch Umsetzung von p-Chinonen mit o-Diaminen. *Mh. Chem.* **90**(6), 827-838.

Peinemann, S., Blaut, M. and Gottschalk, G. (1989). ATP synthesis coupled to methane formation from methyl-CoM and H_2 catalyzed by vesicles of the methanogenic bacterial strain Gö1. *Eur. J. Biochem.* **186**(1-2), 175-180.

Peinemann, S., Müller, V., Blaut, M. and Gottschalk, G. (1988). Bioenergetics of methanogenesis from acetate by *Methanosarcina barkeri*. *J. Bacteriol.* **170**(3), 1369-1372.

Pisa, K. Y., Weidner, C., Maischak, H., Kavermann, H. and Müller, V. (2007). The coupling ion in the methanoarchaeal ATP synthases: H(+) vs. Na(+) in the A(1)A(o) ATP synthase from the archaeon *Methanosarcina mazei* Gö1. *FEMS Microbiol. Lett.* **277**(1), 56-63.

Ragsdale, S. W. (2008). Catalysis of methyl group transfers involving tetrahydrofolate and B12. *Vitam. Horm.* **79**, 293-324.

Ragsdale, S. W., Ljungdahl, L. G. and Der Vartanian, D. V. (1983). Isolation of carbon monoxide dehydrogenase from *Acetobacterium woodii* and comparison of its properties with those of the *Clostridium thermoaceticum* enzyme. *J. Bacteriol.* **155**(3), 1224-1237.

Thauer, R. K. (1998). Biochemistry of methanogenesis: a tribute to Marjory Stephenson. *Microbiology* **144**, 2377-2406.

Thauer, R. K., Jungermann, K. and Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41**(1), 100-180.

Thauer, R. K., Kaster, A. K., Seedorf, H., Buckel, W. and Hedderich, R. (2008). Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* **6**(8), 579-591.

Vignais, P. M., Billoud, B. and Meyer, J. (2001). Classification and phylogeny of hydrogenases. *FEMS Microbiol. Rev.* **25**(4), 455-501.

Welte, C., Kallnik, V., Grapp, M., Bender, G., Ragsdale, S. and Deppenmeier, U. (2010a). Function of Ech hydrogenase in ferredoxin-dependent, membrane-bound electron transport in *Methanosarcina mazei*. *J. Bacteriol.* **192**(3), 674-678.

Welte, C., Krätzer, C. and Deppenmeier, U. (2010b). Involvement of Ech hydrogenase in energy conservation of *Methanosarcina mazei*. *FEBS J.* **277**, 3396-3403.

Wolfe, R. S. (1985). Unusual coenzymes of methanogenesis. *Trends Biochem. Sci.* **10**(10), 396-399.

Zahler, W. L. and Cleland, W. W. (1968). A specific and sensitive assay for disulfides. *J. Biol. Chem.* **243**(4), 716-719.

Zehnder, A. J. B. and Wuhrmann, K. (1976). Titan(III) citrate a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. *Science* **194**, 1165-1166.

Chapter 7
Concluding discussion

Methylotrophic energy conservation in *Methanosarcina* sp. with *Methanosarcina* (*Ms.*) *mazei* as one of the important model organisms is well investigated. Two proton-translocating membrane-bound oxidoreductase systems have been described, the H_2: heterodisulfide oxidoreductase (Ide *et al.*, 1999) and the $F_{420}H_2$: heterodisulfide oxidoreductase (Bäumer *et al.*, 2000). A third heterodisulfide oxidoreductase, the ferredoxin: heterodisulfide oxidoreductase, was proposed to be responsible for ferredoxin turnover in aceticlastic methanogenesis (Fischer and Thauer, 1990). In 1999 Ech hydrogenase was identified as an electron input protein of the ferredoxin: heterodisulfide oxidoreductase in *Ms. barkeri* (Meuer *et al.*, 1999) and it was hypothesized that ferredoxin oxidation and H_2 production are coupled to energy conservation. However, no enzymatic system for continuous reduction of ferredoxin was established and the idea of ion translocation by Ech hydrogenase was not proven by biochemical experiments.

Enzymatic reduction of ferredoxin

The first aim of this work was to establish an enzymatic system with which to analyze ferredoxin-dependent electron transport using isolated cytoplasmic membranes and vectorial ion translocation using membrane vesicles of *Ms. mazei*. This enzymatic system should enable electron transport from an initial substrate to a ferredoxin-reducing enzyme, then to a ferredoxin and finally to the membrane-bound ferredoxin: heterodisulfide oxidoreductase. The *Ms. mazei* genome codes for approximately 20 ferredoxins, and it is not known which of these ferredoxins interacts with the membrane-bound ferredoxin: heterodisulfide oxidoreductase. Initial attempts to produce some of these ferredoxins recombinantly in *Escherichia coli* were not successful due to incomplete assembly of the [4Fe4S] clusters in *E. coli* or *in vitro* reconstitution. As a substitute the [4Fe4S] ferredoxin from *Clostridium pasteurianum* was used because direct purification from the parent organism ascertains correct cofactor assembly. Furthermore, ferredoxin from *C. pasteurianum* is used as model ferredoxin in many studies (Biegel and Müller, 2010; Kaster *et al.*, 2011) and it has been observed that ferredoxins are often functionally interchangeable albeit only distantly related (Tagawa and Arnon, 1968).

In anaerobic metabolism, many enzymes interact with ferredoxins. Unfortunately, many of these are highly oxygen unstable (e.g. CO dehydrogenases), contain unusual cofactors (e.g. the tungsten cofactor of aldehyde: ferredoxin

oxidoreductases) or are built up by many subunits (multi-subunit pyruvate: ferredoxin oxidoreductases) making recombinant production in *E. coli* difficult. Laborious work to purify ferredoxin-reducing enzymes directly from anaerobic organisms was inevitable, and thankfully a multi-subunit CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) from *Moorella thermoacetica* was provided by Güneş Bender and Steve Ragsdale (University of Michigan). The CODH/ACS couples CO oxidation to ferredoxin reduction, and indeed worked productively with the ferredoxin from *C. pasteurianum*. This ferredoxin, in turn, served as electron donor to the *Ms. mazei* membrane-bound ferredoxin: heterodisulfide oxidoreductase, and led to heterodisulfide reduction. With this system, electron transport of the ferredoxin: heterodisulfide oxidoreductase system could be followed, and also the activity of Ech hydrogenase alone could be continuously measured.

Revealing energy-transducing properties of Ech hydrogenase

Ferredoxin-dependent electron transport measurements with *Ms. mazei* wildtype and *Ms. mazei* Δ*ech* mutant membrane preparations revealed that the heterodisulfide reduction rate was strongly decreased in the Δ*ech* mutant. This finding confirmed the proposed involvement of Ech hydrogenase in the ferredoxin: heterodisulfide oxidoreductase and validated the proposed hydrogen-cycling mechanism in which Ech hydrogenase couples ferredoxin oxidation to hydrogen production. Hydrogen is taken up by F_{420} non-reducing hydrogenase that donates electrons to the methanophenazine pool. Reduced methanophenazine finally acts as substrate to the heterodisulfide reductase. It is important to mention that membrane-bound ferredoxin: heterodisulfide oxidoreductase activity was not abolished in the *Ms. mazei* Δ*ech* mutant but was still present to an extent of about 50 %. This makes the existence of a second ferredoxin-interacting membrane protein (complex) likely.

The phenotypic characterization of the *Ms. mazei* Δ*ech* mutant showed that growth on methylated amines was slower, substrate consumption was faster and the overall growth yield was lower than observed for the wildtype. Furthermore, the Δ*ech* mutant did not grow on acetate. Taking together it became evident that substrate usage was less effective in the mutant than in the wildtype, indicating an important function of Ech hydrogenase in energy conservation. However, reduced ferredoxin that is produced in methylotrophic methanogenesis had to be re-oxidized in the Δ*ech* mutant in a process that was obviously only possible with energetic disadvantages. There are two possibilities how the cell could accomplish the reoxidation of reduced ferredoxin in the absence of the Ech hydrogenase: (i) there

could be a soluble ferredoxin: F_{420} oxidoreductase that transfers electrons from reduced ferredoxin to coenzyme F_{420} and finally to the F_{420}-dependent electron transport pathway. (ii) There could be an Ech hydrogenase-independent membrane-bound ferredoxin: heterodisulfide oxidoreductase system present that conserves less energy than the Ech-dependent pathway.

To further address the question of Ech hydrogenase as an energy-conserving enzyme, a vesicular system of inside-out oriented membrane vesicles was employed. Enzymatic activities that normally lead to the formation of an electro-chemical ion gradient over the cytoplasmic membranes result in an accumulation of the respective ions in the lumen of the inside-out oriented membrane vesicles. The A_1A_O ATP synthase of *Ms. mazei* can take advantage of this potential and generate ATP from ADP + P_i through reflux of H^+ or Na^+ ions. Experiments with membrane vesicles of the *Ms. mazei* wildtype showed that the activity of Ech hydrogenase was correlated with ATP production. This ATP synthesis was strictly dependent on an ion gradient because the inhibitor DCCD abolishes ATP formation. These data showed that Ech hydrogenase is an ion-translocating enzyme that conserves energy. In methanogenic bioenergetics both H^+ and Na^+ serve as coupling ions so the nature of the coupling ion of Ech hydrogenase had to be explored. The sodium ionophore ETH157 did not alter ATP formation through Ech hydrogenase activity, but the protonophore SF6847 effectively prevented ATP synthesis. At the same time the activity of Ech hydrogenase was monitored following ferredoxin-dependent H_2 production and it was found that Ech hydrogenase activity was not altered by the ionophores. Theoretically a maximum of 1 H^+ per 2 e^- can be transported across the membrane. Energy conservation by Ech hydrogenase is in accordance with previous hypotheses (Bott and Thauer, 1989; Meuer *et al.*, 1999; Tersteegen and Hedderich, 1999; Stojanowic and Hedderich, 2004). Also the related Mbh hydrogenase of *Pyrococcus furiosus* was shown to couple ferredoxin oxidation to proton reduction and ion movement over the cytoplasmic membrane (Sapra *et al.*, 2003). In summary, it was clearly shown for the first time by experimental data that the Ech hydrogenase from *Methanosarcina* sp. belongs to the group of energy-conserving hydrogenases.

Pathway of respiratory $F_{420}H_2$ turnover

Hydrogen cycling by the *Ms. mazei* and *Ms. barkeri* ferredoxin: heterodisulfide oxidoreductase initially seemed unlikely because a gaseous intermediate could easily escape the respiratory system and would lead to energy loss. But apparently these methanogens effectively capture the gaseous H_2, so the overall partial H_2

pressure of acetate-growing cultures is low (Lovley and Ferry, 1985). However, hydrogen cycling was not thought to be involved in energy conservation for the *Ms. mazei* F_{420}: heterodisulfide oxidoreductase because the membrane-bound $F_{420}H_2$ dehydrogenase directly channels electrons to methanophenazine. Nevertheless, the possibility of an alternative soluble F_{420} hydrogenase that couples $F_{420}H_2$ oxidation to hydrogen production exists and could be utilized in an analogous hydrogen cycling mechanism as observed for ferredoxin oxidation. For the close relative *Ms. barkeri* hydrogen cycling in the course of heterodisulfide-dependent $F_{420}H_2$ oxidation was proposed to be the predominant mechanism (Kulkarni *et al.*, 2009). Taking these observations together, a re-investigation of the $F_{420}H_2$ oxidizing mechanism in *Ms. mazei* and close relatives was done with the aim of resolving the pathway by which electrons from $F_{420}H_2$ enter the respiratory chain. The phenotypic characterizations of two mutants of the $F_{420}H_2$ dehydrogenase (*Ms. mazei* Δ*fpoA-O* and Δ*fpoF*) revealed that the deletion of the $F_{420}H_2$ dehydrogenase leads to severely slowed growth on methylated amines comparable to growth of the *Ms. mazei* Δ*ech* mutant. However, the growth yield was not affected, indicating that the F_{420} hydrogenase system can energetically substitute for the loss of $F_{420}H_2$ dehydrogenase. Measurements of the $F_{420}H_2$: heterodisulfide oxidoreductase activity in cell lysate demonstrated that this activity is greatly reduced in the two Δ*fpo* mutants, and also the methane formation rates of resting cell suspensions was strongly decreased. In summary, these findings showed that only a minor part of $F_{420}H_2$ oxidation is performed by F_{420} hydrogenase using hydrogen cycling in *Ms. mazei*. Other *Methanosarcina* sp. including *Ms. acetivorans* seem to be similar in this preference, and *Ms. barkeri* can be regarded as an exception that prefers the use of F_{420} hydrogenase.

FpoF, a novel ferredoxin: F_{420} oxidoreductase

Membrane fractions of the *Ms. mazei* Δ*fpoF* mutant, missing the electron input subunit FpoF of the $F_{420}H_2$ dehydrogenase, were impaired in their ability to oxidize $F_{420}H_2$.The same result was observed for the Δ*fpoA-O* mutant that lacked the membrane-integral and membrane-associated part of $F_{420}H_2$ dehydrogenase. It was surprising that the cytoplasmic activities of the two mutant strains were different. The cytoplasm of the parental strain was found to catalyze the redox-reaction of ferredoxin with F_{420} by use of a novel ferredoxin: F_{420} oxidoreductase. The cytoplasm of the Δ*fpoA-O* mutant retained similar levels of activity whereas the cytoplasmic fraction of the Δ*fpoF* mutant could no longer catalyze the ferredoxin-dependent redox reaction. To investigate this enzymatic activity in more detail the FpoF protein

was recombinantly produced in *E. coli*. The purified protein catalyzed the ferredoxin: F_{420} oxidoreductase reaction with high specificity but low rates, and was found to be present in substantial quantities as soluble protein in the cytoplasm. It can be concluded that FpoF indeed catalyzes the electron transfer between ferredoxin and F_{420}. However, this connection has to be strongly regulated because reduction of F_{420} with reduced ferredoxin results in a decreased H^+ / e^- ratio. This is evident from the fact that the ferredoxin: heterodisulfide oxidoreductase translocates 5 H^+ / 2 e^- (with Ech hydrogenase as proton-translocatig enzyme) whereas the $F_{420}H_2$: heterodisulfide oxidoreductase only translocates 4 H^+ / 2 e^-.

In principle, the discovery of the ferredoxin: F_{420} oxidoreductase could explain the survival of the *Ms. mazei* Δ*ech* mutant, because reduced ferredoxin that is produced in the methylotrophic pathway could be re-oxidized by FpoF and the electrons could be used by the $F_{420}H_2$: heterodisulfide oxidoreductase. However, the FpoF activity (also in the cytoplasm of the Δ*ech* mutant) is too low to allow effective turnover of reduced ferredoxin, and can only partially contribute to the survival of the Δ*ech* mutant. Hence, the existence of another membrane-bound ferredoxin-oxidizing enzyme is still a tempting alternative.

Membrane-bound electron transport in *Methanosaeta thermophila*

Aceticlastic energy conservation in *Ms. mazei* was investigated in detail in the course of this work. However, the energy conserving system of the close relative *Methanosaeta* (*Mt*.) which is the only obligately aceticlastic methanogenic genus has not been examined to date. Comparative studies of the *Mt. thermophila* genome indicated that protein complexes involved in electron input to the aceticlastic respiratory chain of *Methanosarcina* sp. are absent: neither genes coding for Ech hydrogenase nor for the Rnf complex can be found in the genome. Furthermore, the genome does not contain intact hydrogenase genes, and also only an incomplete coding region for the $F_{420}H_2$ dehydrogenase that lacks the electron input protein FpoF. Genes coding for a membrane-bound heterodisulfide reductase (HdrDE) were found and activity measurements on isolated *Mt. thermophila* membranes showed that the corresponding enzyme is active and exhibits comparable activities to the *Ms. mazei* enzyme. This means that energy conservation via a membrane-bound heterodisulfide oxidoreductase system is likely. $F_{420}H_2$, NAD(P)H and H_2 did not serve as electron donors to the membrane. The recombinant *Ms. mazei* ferredoxin MM1619 as well as clostridial ferredoxin (not shown) served as electron donors for heterodisulfide reduction, so a functional ferredoxin: heterodisulfide oxidoreductase system is present in *Mt. thermophila*. The rate of membrane-bound heterodisulfide

reduction was two- to three-fold faster in *Mt. thermophila* than in acetate-grown *Ms. mazei* which makes it likely that the *Mt. thermophila* ferredoxin: heterodisulfide oxidoreductase is actually used for energy generation in the obligate aceticlastic organism.

In *Mt. thermophila*, the main part of the $F_{420}H_2$ dehydrogenase is encoded in the genome (*fpoABCDHIJKLMN*), but the electron input subunit FpoF is missing. The presence of genes encoding the $F_{420}H_2$ dehydrogenase in combination with the apparent inability of the *Mt. thermophila* membranes to oxidize $F_{420}H_2$ resulted in the idea that the incomplete $F_{420}H_2$ dehydrogenase could be involved in ferredoxin-dependent electron transport. Two possibilities concerning how the $F_{420}H_2$ dehydrogenase could contribute to ferredoxin oxidation exist: (i) in *Mt. thermophila*, there exists another unknown electron input subunit that productively works with FpoA-N and is located elsewhere in the chromosome as is also the case for the *fpoF* gene in *Methanosarcina* sp. FpoA-N is highly homologous to the core of complex I (NADH dehydrogenase I of *E. coli*, Nuo), and the replacement of NuoEFG by FpoF enables methanogens to use $F_{420}H_2$ instead of NADH. Another replacement of the electron input module together with the core Fpo/Nuo could be a feasible possibility to enable ferredoxin oxidation. (ii) The core enzyme FpoA-N is catalytically active without FpoF or other subunits channelling electrons immediately into the respiratory chain. Hence, the truncated Fpo complex would accept electrons directly from reduced ferredoxin without the need of an alternative electron input subunit.

To address these questions, the diversity and distribution of complex I homologues is discussed. Complex I from *E. coli* is termed **N**ADH: **u**biquinone **o**xidoreductase (Nuo) that has a homologous subunit nomenclature to the *Ms. mazei* **F**$_{420}$H$_2$: **p**henazine **o**xidoreductase (Fpo). Other terminologies include the **N**ADH: **q**uinone **o**xidoreductase of *Thermus thermophilus* (Nqo) or **N**ADH **d**e**h**ydrogenase **1** (NDH-1) of cyanobacteria. For simplicity, the Nuo nomenclature will be used throughout this chapter.

NADH dehydrogenases in bacteria

NADH dehydrogenases are widely distributed among living organisms, and are well investigated in mitochondria of eukaryotic cells and eubacteria. Only recently, the crystal structure of the complete *E. coli* and *T. thermophilus* NADH dehydrogenase (complex I) has been solved (Efremov *et al.*, 2010). The bacterial NADH dehydrogenase contains four proton coupling sites within its 14 subunits. These are

organized in three functional modules (Friedrich and Scheide, 2000). The first is the central membrane-integral and membrane-associated NuoBCDHIL that probably has a common ancestor with the energy-conserving hydrogenase family (e.g. Ech hydrogenase). NuoBCDHIL lost the hydrogenase [NiFe] site and acquired a quinone-binding site, and accounts for one coupling site. The subunit responsible for proton translocation is NuoL (Efremov *et al.*, 2010; Nakamaru-Ogiso *et al.*, 2010; Ohnishi *et al.*, 2010) that shows high homologies to Na^+ or K^+/H^+ antiporters (Fearnley and Walker, 1992; Friedrich and Weiss, 1997; Mathiesen and Hägerhäll, 2002). Homologues of NuoL are also found in the *Ms. mazei* Ech hydrogenase (EchA) and $F_{420}H_2$ dehydrogenase (FpoL) and are probably also involved in proton translocation. The second module comprises NuoAJKMN and accounts for two coupling sites of the NADH dehydrogenase. Gene triplication of *nuoL* gave rise to *nuoMN* whose gene products are involved in proton translocation of the second module (Torres-Bacete *et al.*, 2007; Euro *et al.*, 2008; Torres-Bacete *et al.*, 2009). The exact mechanism for the coupling of the fourth proton is not known (Efremov *et al.*, 2010). The third module NuoEFG is responsible for NADH oxidation and the flavin-based 2 e^- / 1 e^- transfer from NADH to the iron-sulfur clusters (Figure 4). It shows homologies to the diaphorase subunit of the NAD^+-reducing hydrogenase of *Cupriavidus necator* (Tran-Betcke *et al.*, 1990; Pilkington *et al.*, 1991; Albracht, 1993; Massanz *et al.*, 1998).

In *E. coli* and other eubacteria, *nuoEFG* is encoded in the same operon as the other *nuo* genes, whereas in the early off-branching eubacterium *Aquifex aeolicus*, *nuoEFG* is located at a different chromosomal locus (Deckert *et al.*, 1998). Obviously the acquirement of the *nuoEFG* genes was independent of the development of NuoABCDHIJKLMN. Hence, it is possible that the partial complex without NuoEFG has a function of its own. It has been hypothesized that the NuoEFG-independent Nuo worked as donor: quinone oxidoreductase or more precisely as ferredoxin: quinone oxidoreductase (Friedrich *et al.*, 1995). In early life, the atmosphere was oxygen-free and the abundance of iron and sulfur allowed the formation of highly redox-negative iron-sulfur clusters (Wächtershäuser, 1988; Imlay, 2006). Simple oxidoreductase systems might have used ferredoxin, a small acidic protein containing an iron-sulfur cluster, as redox carrier (Hall *et al.*, 1971; George *et al.*, 1985; Imlay, 2006; Ma *et al.*, 2008a). Furthermore, ferredoxins are thought to be among the most ancient proteins (George *et al.*, 1985). Thus, ancestors of the core NuoABCDHIJKLMN might have interacted with ferredoxin, and released the electrons into the membrane. Later the atmosphere became oxygenic, and fragile iron-sulfur clusters had to be replaced (Imlay, 2006). Then the oxygen-

stable NADH started to become important as a redox carrier. It is feasible to assume that by that time NuoABCDHIJKLMN acquired NuoEFG. The acquisition of NuoEFG allowed the partial replacement of the oxygen-labile ferredoxin as redox carrier and the dynamic shift to aerobiosis and oxygen tolerance.

Exceptions to the bacterial "NADH dehydrogenases"

Although the Nuo complex containing NuoEFG seems to be the predominant form of the Nuo complex in eubacteria, there are exceptions. Nuo complexes of ε-proteobacteria and cyanobacteria do not contain the classical electron input module NuoEFG, and alternative electron input modules and electron donors replacing NADH are to be discussed.

In ε-proteobacteria, *nuoE* and *nuoF* are absent, and *nuoG* is modified (Smith *et al.*, 2000; Weerakoon and Olson, 2008). It is not surprising that the ε-proteobacterium *Campylobacter jejuni* does not exhibit membrane-bound NADH oxidase activity due to the lack of the NADH-binding NuoF subunit (Hoffman and Goodman, 1982; Weerakoon and Olson, 2008). Instead, *C. jejuni* and the close relative *Helicobacter pylori* contain additional genes in the *nuo* operon (*cj1574c* and *cj1575c*, Tomb *et al.*, 1997; Finel, 1998; Parkhill *et al.*, 2000; Smith *et al.*, 2000) whose gene products are thought to complement the truncated Nuo complex in these organisms (Weerakoon and Olson, 2008). This alternative Nuo complex is thought to accept electrons from flavodoxin (Figure 4, Weerakoon and Olson, 2008). Flavodoxins are small acidic proteins resembling ferredoxins that additionally contain FMN (Sancho, 2006). It is noteworthy that amino acids are important substrates for *C. jejuni* and *H. pylori* and that central amino acid degrading enzymes have unusual properties. Pyruvate dehydrogenase is replaced by a pyruvate: flavodoxin oxidoreductase (Hughes *et al.*, 1995; Hughes *et al.*, 1998; Parkhill *et al.*, 2000) and α-ketoglutarate dehydrogenase is replaced by α-ketoglutarate: flavodoxin oxidoreductase (Hughes *et al.*, 1998; Parkhill *et al.*, 2000; Weerakoon and Olson, 2008). Overall, the alteration of complex I in *H. pylori* und *C. jejuni* from an NADH: quinone oxidoreductase to a flavodoxin: quinone oxidoreductase is mirrored in the central donor oxidoreductases. In *Ms. mazei* and *Mt. thermophila*, homologues to the flavodoxin-oxidizing electron input module cannot be found, and flavodoxin-interacting oxidoreductases have not been described. Thus, a flavodoxin interaction of the truncated Nuo/Fpo in *Mt. thermophila* or *Ms. mazei* is unlikely.

The second prominent eubacterial group that contains only incomplete nuo operons are the cyanobacteria. Chloroplasts and cyanobacteria contain *nuo* gene clusters

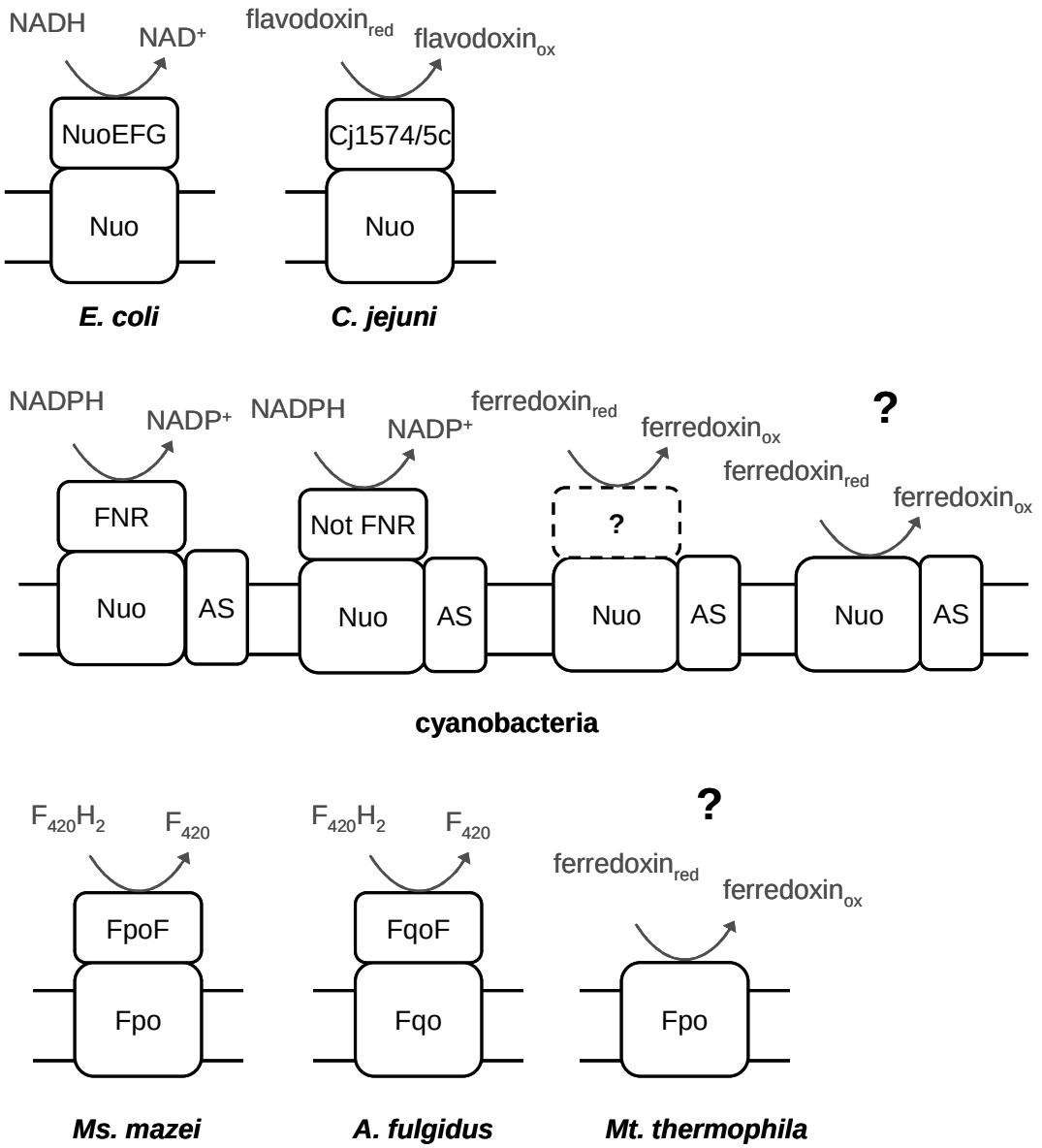

Figure 4: Simplified model of the functioning of Nuo homologues in different bacteria and archaea. The *E. coli* electron input module NuoEFG is replaced by other known or unknown electron input modules. In a cyanobacterial Nuo isozyme and the *Mt. thermophila* Nuo-homologue Fpo, a distinct electron input module might be absent. Question marks indicate unknown reactions or subunit(s). AS, additional subunits; FNR, ferredoxin-$NADP^+$ reductase.

that lack *nuoEFG* and unsurprisingly NADH: quinone oxidoreductase activity cannot be found in the respective cytoplasmic or thylakoid membranes (Battchikova and Aro, 2007). Furthermore, these organisms do not contain homologues to the flavodoxin-oxidizing subunit(s) of the Nuo complex of *H. pylori* and *C. jejuni*. In the cyanobacterium *Synechocystis*, the *nuo* genes are scattered across the chromosome (Kaneko and Tabata, 1997). In-depth investigations revealed that the *Synechocystis* Nuo complex is present in multiple isoforms (Figure 4, Battchikova *et al.*, 2011) with additional subunits that are not coded together with the *nuo* genes

(Prommeenate *et al.*, 2004; Zhang *et al.*, 2004; Battchikova *et al.*, 2005; Rumeau *et al.*, 2005; Nowaczyk *et al.*, 2011). The cyanobacterial complex I isozymes seem to fulfil various functions including roles in respiration, cyclic electron flow and CO_2 uptake (Shibata *et al.*, 2001; Battchikova and Aro, 2007). There is no consensus about the electron donor or the electron input subunit(s) of the cyanobacterial Nuo isozymes (Battchikova *et al.*, 2011).

However, for one complex I homologue it has been shown that NADPH serves as electron donor (Figure 4, (Ma *et al.*, 2006; Ma *et al.*, 2008b) whereas for other cyanobacterial Nuo complexes it was demonstrated that NADPH is not involved (Prommeenate *et al.*, 2004; Battchikova *et al.*, 2011). Some studies identify the electron input module of a NADPH-oxidizing Nuo complex as ferredoxin-$NADP^+$ reductase (FNR, Guedeney *et al.*, 1996; Funk *et al.*, 1999). In contrast, other groups find FNR separated from the NADPH-oxidizing Nuo complex (Ma *et al.*, 2006).

For some cyanobacterial or chloroplastid Nuo complexes, ferredoxin is hypothesized to be the electron donor (Friedrich *et al.*, 1995; Mi *et al.*, 1995; Battchikova *et al.*, 2011). Either ferredoxin interacts with the cyanobacterial Nuo complex without an electron input module as it is now hypothesized for the *Mt. thermophila* Fpo/Nuo complex or additional proteins are involved in the binding of ferredoxin (Figure 4, Prommeenate *et al.*, 2004; Battchikova *et al.*, 2011). Furthermore, there are indications that photosystem I and the Nuo complex form a supercomplex in chloroplasts with direct electron transfer (Peng *et al.*, 2008; Sirpiö *et al.*, 2009).

Overall, the composition of the cyanobacterial Nuo complex isozymes is highly diverse. For some isozymes the replacement of NuoEFG by another electron input module has been demonstrated, whereas the electron input module and cofactor reactivity of other isozymes remains enigmatic. A distinct ferredoxin-oxidizing Nuo complex isozyme has been hypothesized but it has not been experimentally validated, so an analogy to the possibly ferredoxin-interacting Nuo/Fpo complex of *Mt. thermophila* is not possible.

“NADH dehydrogenases” in archaea

In archaea, Nuo homologues have hardly been investigated. Archaeal genomes always lack *nuoEFG* in their *nuo* gene clusters that are nevertheless widely distributed. *Sulofolobus solfataricus* (She *et al.*, 2001), *Thermoplasma acidophilum (Ruepp et al., 2000)*, *Halobacterium salinarum* (Ng *et al.*, 2000), *Aeropyrum pernix* (Kawarabayasi *et al.*, 2001) as well as various members of the *Methanosarcinales* (*Ms. mazei*, *Ms. barkeri*, *Mt. thermophila*) and the close relative *Archaeoglobus*

fulgidus (Klenk *et al.*, 1997) contain homologues of *nuo* genes. Obviously the evolutionary event of the acquisition of *nuoEFG* happened after the separation of eubacteria and archaea. The central questions are: (i) Is the archaeal Nuo complex connected to a phylogenetically unrelated but functionally equivalent electron input subunit to NuoEFG? (ii) Does the archaeal Nuo work without an explicit electron input subunit as "head-less", truncated NuoABCDHIJKLMN? (iii) What is the electron donor to the archaeal Nuo?

For *Methanosarcina* sp. and *A. fulgidus* these questions can be answered (Figure 4). It has been found that an alternative electron input module, FpoF or FqoF, is used that is specialized for the oxidation of $F_{420}H_2$, and the whole enzyme is called $F_{420}H_2$: phenazine oxidoreductase (Fpo) in the case of methanogens, and $F_{420}H_2$: quinone oxidoreductase (Fqo) in the case of *A. fulgidus* (Kunow *et al.*, 1994). *Methanosarcina* sp. encode the *fpoF* gene at a different chromosomal locus than the *fpoA-O* operon (Deppenmeier *et al.*, 2002) whereas *A. fulgidus* contains all genes in one operon (Klenk *et al.*, 1997). Friedrich and Scheide (2000) attempted to classify the archaeal Nuo as $F_{420}H_2$: quinone/phenazine oxidoreductase, with FpoF/FqoF replacing NuoEFG and $F_{420}H_2$ as ubiquitous electron donor instead of NADH. Regarding the distribution of FpoF/FqoF and $F_{420}H_2$ metabolism among archaea this hypothesis seems too generalized. FpoF/FqoF homologues can exclusively be found in methanogens and *Archaeoglobales*. An involvement of $F_{420}H_2$ in archaeal bioenergetics was only shown for the abovementioned orders. This is in accordance with measurements of the cellular F_{420} content of these archaea. For methanogens an F_{420} content of 0.5 – 4 nmol mg protein^{-1} was measured (Eirich *et al.*, 1979; Schönheit *et al.*, 1981; van Beelen *et al.*, 1983; Peck, 1989) and *A. fulgidus* is reported to contain 0.6 nmol F_{420} mg protein^{-1} when grown on lactate and <0.03 nmol mg protein^{-1} when grown on starch (Gorris *et al.*, 1991; Hartzell and Reed, 2006) which bypasses F_{420} metabolism. Halophilic archaea contain a moderate (0.2 – 0.4 nmol mg protein^{-1} for *Haloarcula marismortui*, *Haloferax volcanii*, *Haloarcula* GN-1) to low (0.05 – 0.1 nmol mg protein^{-1} for *Halobacterium salinarum*, *Halococcus morrhuae*) level of F_{420} (Lin and White, 1986) and an involvement of F_{420} in energy conservation has not been suggested. F_{420} is probably needed for biosynthetic reactions as it was noted for the non-related actinobacteria (Selengut and Haft, 2010) that contain 0.050 – 0.1 nmol F_{420} mg protein^{-1} (Lin and White, 1986). Nevertheless it is noteworthy that halophilic and methanogenic archaea as well as the *Archaeoglobales* belong to the kingdom Euryarchaeota whereas members of the kingdom Crenarchaeota (*Sulfolobus solfataricus*, *Thermoproteus tenax*) barely contain F_{420} (Lin and White, 1986; Noll and Barber,

1988). It is apparent that the bioenergetical use of F_{420} is very limited among the archaea, and that the designation Fqo/Fpo for archaeal Nuo homologues is only warranted for the specialized groups of methanogens and the *Archaeoglobales* containing FpoF/FqoF.

For the other archaea, however, no electron input protein has been found and the functioning of Nuo homologues remains unclear. In light of the nature of the electron donor to the respective respiratory systems it is noteworthy that ferredoxin is ubiquitous in archaea and that many archaea have replaced bacterial NADH-producing enzymes with ferredoxin-dependent enzymes. Biochemical investigations revealed a huge variety of 2-oxoacid: ferredoxin oxidoreductases (Kerscher and Oesterhelt, 1981; Mai and Adams, 1996; Tersteegen *et al.*, 1997; Nishizawa *et al.*, 2005; Puchkaev and Ortiz de Montellano, 2005; Park *et al.*, 2006) as well as aldehyde: ferredoxin oxidoreductases (George *et al.*, 1992; Mukund and Adams, 1993; Roy *et al.*, 1999; Roy and Adams, 2002; Hagedoorn *et al.*, 2005) and glyceraldehyde: ferredoxin oxidoreductase in archaea (Mukund and Adams, 1995; Selig *et al.*, 1997; van der Oost *et al.*, 1998; Labes and Schönheit, 2001; Sakuraba and Ohshima, 2002; Park *et al.*, 2007; Reher *et al.*, 2007). The Nuo complex performs membrane-bound NADH oxidation in most bacteria and eukaryotes. In archaea, however, NADH is less important and often replaced by ferredoxin. In analogy to the bacterial Nuo complex, a ubiquitous ferredoxin-oxidizing membrane protein (complex) was expected in archaea but was not identified.

Is the archaeal “NADH dehydrogenase” a ferredoxin-oxidizing enzyme?

It seems feasible that the truncated Nuo complex serves as ferredoxin: quinone oxidoreductase, at least in archaeal bioenergetics. This is consistent with the hypothesis that *Mt. thermophila* uses the truncated Nuo-homologue Fpo for ferredoxin oxidation. Weak homologies of FpoD with the quinone-interacting NuoD allow the organism to transfer electrons to methanophenazine instead of quinone as already shown for the *Ms. mazei* enzyme (Bäumer *et al.*, 2000). Ferredoxin could transfer electrons to the iron-sulfur clusters in either FpoB or FpoI. When FpoI paralogues of different *Methanosarcina* and *Methanosaeta* sp. are compared (Figure 5) it becomes apparent that the FpoI subunit of both *Methanosaeta harundinacea* and *Mt. thermophila* is modified: in these organisms FpoI contains a C-terminal extension with an accumulation of lysine residues. Lysine is a basic amino acid and the basic C-terminal extension could facilitate the interaction with the acidic ferredoxin.

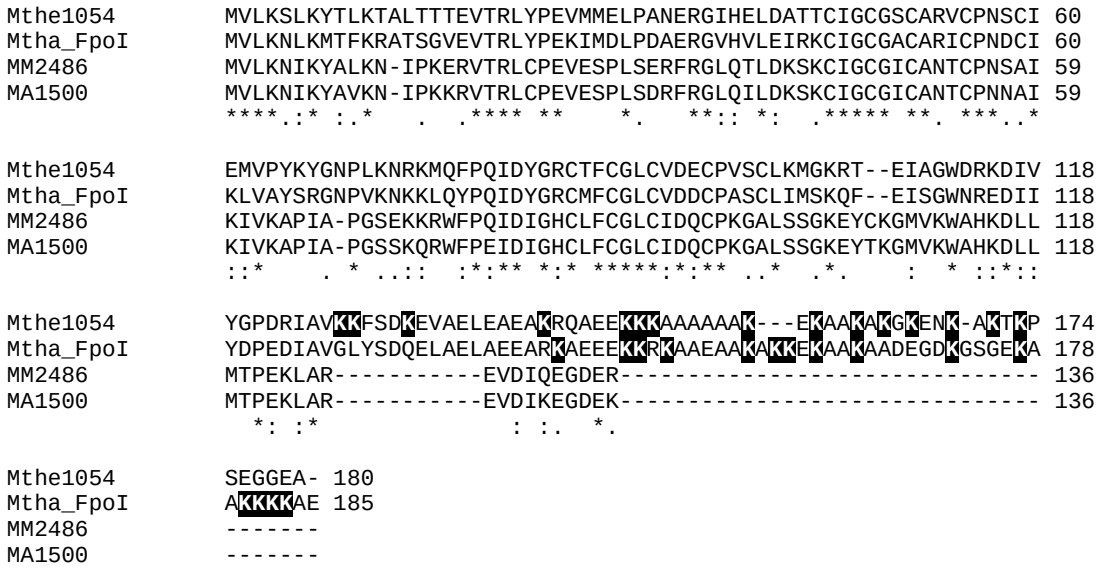

Figure 5: Alignment of FpoI homologues of different methanogenic archaea. FpoI of *Methanosaeta* sp. contains a C-terminal extension with an accumulation of basic lysine residues. Mthe1054, *Mt. thermophila* YP_843478.1; Mtha_FpoI, *Mt. harundinacea* ADQ42349.1; MM2486, *Ms. mazei* NP_634510.1 (the corresponding gene probably contains a wrong starting point in the database. In the alignment, an alternative starting point further downstream was chosen); MA1500, *Ms. acetivorans* NP_616434.1.

When the hypothesis of the truncated Fpo acting as ferredoxin: phenazine oxidoreductase is applied to ferredoxin-dependent electron transport of *Ms. mazei* it becomes apparent that the residual ferredoxin-oxidizing activity of the *Ms. mazei* Δ*ech* mutant could be due to the activity of the truncated Fpo. Under normal conditions ferredoxin oxidation by Fpo is prevented by binding of FpoF because ferredoxin oxidation by Ech hydrogenase is energetically favoured (5 H^+ versus 4 H^+ / 2 e^-). In contrast, *Mt. thermophila* does not contain hydrogenases and still might be dependent on the truncated Nuo/Fpo for ferredoxin re-oxidation. The pathway utilized by *Mt. thermophila* probably gains 4 H^+ / 2 e^- whereas the hydrogen cycling pathway used by *Ms. mazei* results in 5 H^+ / 2 e^-. The decrease of efficiency of energy conservation in *Mt. thermophila* has to be compensated by economization of the substrate uptake and/or activation pathway that has yet to be discovered.

Overall, the truncated complex I (Nuo/Fpo/Fqo) that lacks NuoEFG/FpoF/FqoF might be a central enzyme in bioenergetics and energy conservation of *Methanosaeta* sp.. In *Methanosarcina* sp., the truncated Fpo might have a less important role in ferredoxin oxidation when the primary ferredoxin-interacting Ech hydrogenase is absent (e.g. in the *Ms. mazei* Δ*ech* mutant) or inhibited (e.g. by high concentrations of CO (Meuer *et al.*, 1999)). Furthermore, an involvement of the truncated Nuo/Fqo in archaeal bioenergetics is likely. Moreover, ferredoxin-

dependent electron transport might be of greater importance than previously thought.

Chapter 8
Summary

1) Aceticlastic and methylotrophic methanogens produce reduced ferredoxin in the course of methane formation that is re-oxidized by membrane-bound enzymes. To study ferredoxin-dependent electron transport processes, a defined system for continuous generation of reduced ferredoxin was established. The CO-oxidizing CO dehydrogenase/acetyl-CoA synthase from *Moorella thermoacetica* was used to reduce *Clostridium pasteurianum* ferredoxin or the methanogenic ferredoxin MM1619. This system was successfully used to study different ferredoxin-dependent electron transport pathways.

2) Ferredoxin-dependent heterodisulfide reduction was measured in *Ms. mazei* membrane preparations for the first time in the course of this work. *Ms. mazei* mutants lacking Ech hydrogenase exhibited significantly reduced ferredoxin-dependent electron transport rates by about 50 % in comparison to the wildtype. The involvement of Ech hydrogenase in the ferredoxin: heterodisulfide oxidoreductase system was clearly demonstrated. Furthermore, a second membrane-bound protein responsible for the residual 50 % ferredoxin-dependent heterodisulfide reduction was postulated.

3) The *Ms. mazei* Δ*ech* mutant exhibited a pronounced phenotype with respect to growth parameters. It showed slower growth, less biomass yield and accelerated substrate consumption when grown on trimethylamine. Furthermore, the mutant did not grow on acetate. These findings underlined a possible energy conserving function of Ech hydrogenase. In addition, it became evident that a second ferredoxin-oxidizing pathway is necessary to explain the survival of the mutant when grown on trimethylamine.

4) To address the question whether Ech hydrogenase belongs to the energy-converting hydrogenase family, experiments with inverted membrane vesicles from *Ms. mazei* were performed. These experiments demonstrated that Ech hydrogenase is able to form molecular hydrogen by the oxidation of reduced ferredoxin. This process was coupled to the translocation of ions over the cytoplasmic membrane and ATP was synthesized by electron transport phosphorylation. The translocated ion was identified as proton and Na^+ ion transport was excluded. Theoretical considerations led to a 1 H^+ / 2 e^- stoichiometry.

5) $F_{420}H_2$ is a reducing equivalent that is produced in hydrogenotrophic and methylotrophic methanogenesis. The oxidation of $F_{420}H_2$ in *Methanosarcina* sp. can

proceed via the membrane-bound $F_{420}H_2$ dehydrogenase (Fpo) or the soluble F_{420} hydrogenase (Frh). Frh produces molecular hydrogen in the course of $F_{420}H_2$ oxidation that is used by the uptake hydrogenase Vho. To find out which pathway is preferred by *Ms. mazei* two mutants of the $F_{420}H_2$ dehydrogenase (Δ*fpoF* and Δ*fpoA-O*) were constructed. Both mutants grew slower than the wildtype on trimethylamine, but exhibited similar growth rates compared to the parental strain when grown on trimethylamine + H_2 or acetate. Enzymatic activity measurements of cell lysates, membranes, and cytoplasmic fractions revealed that the majority of $F_{420}H_2$ oxidation in *Ms. mazei* is performed by $F_{420}H_2$ dehydrogenase and only a minor part by F_{420} hydrogenase.

6) The characterization of the *Ms. mazei fpo* mutants revealed that the deletion of *fpoF* leads not only to an alteration of membrane-bound electron transport but also to a change of enzyme activities in the cytoplasm. The *Ms. mazei* Δ*fpoF* mutant no longer catalyzed the ferredoxin: F_{420} oxidoreductase reaction. Subsequently, FpoF was heterologously produced in *E. coli* and purified to homogeneity. The recombinant FpoF was characterized as ferredoxin: F_{420} oxidoreductase and contained one FAD and two [4Fe4S] clusters per monomer. Quantitative Western blot experiments showed that FpoF is found in the membrane fraction where it is bound to the FpoA-O complex but was also present in substantial quantities in the cytoplasmic fraction.

7) Energy conservation in *Methanosarcina* sp. is by now well investigated, whereas energy conservation pathways of members of the obligate aceticlastic genus *Methanosaeta* remain largely unknown. During this work, membrane-bound electron transport pathways of *Mt. thermophila* were investigated. *Mt. thermophila* was found to possess a highly active membrane-bound ferredoxin: heterodisulfide oxidoreductase. The *Mt. thermophila* genome does not contain genes coding for known ferredoxin-interacting membrane proteins (Ech hydrogenase, Rnf complex). However, it contains genes coding for the $F_{420}H_2$ dehydrogenase (without the electron input subunit FpoF) although the membrane does not interact with $F_{420}H_2$. As indicated in chapter 7, the $F_{420}H_2$ dehydrogenase is highly homologous to the bacterial NADH dehydrogenase I (complex I). Replacements of the electron input module of complex I enables the enzyme to oxidize different electron carriers (NADPH, flavodoxin) in ε-proteobacteria and cyanobacteria. In archaea, NADH dehydrogenase I homologues mostly appear without a distinct electron input module. Hence, it is tempting to speculate that in *Mt. thermophila* the truncated $F_{420}H_2$ dehydrogenase, as a homologue of complex I, is responsible for ferredoxin oxidation, thereby channelling electrons into the respiratory chain.

Reference list

Abken HJ, Tietze M, Brodersen J, Bäumer S, Beifuss U, Deppenmeier U (1998) Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Gö1. *J Bacteriol* **180**: 2027-2032.

Albracht SP (1993) Intimate relationships of the large and the small subunits of all nickel hydrogenases with two nuclear-encoded subunits of mitochondrial NADH: ubiquinone oxidoreductase. *Biochim Biophys Acta* **1144**: 221-224.

Alex LA, Reeve JN, Orme-Johnson WH, Walsh CT (1990) Cloning, sequence determination, and expression of the genes encoding the subunits of the Nickel-containing 8-hydroxy-5-deazaflavin reducing hydrogenase from *Methanobacterium thermoautotrophicum* ΔH. *Biochemistry* **29**: 7237-7244.

Allen GWJ, Zinder SH (1996) Methanogenesis from acetate by cell-free extracts of the thermophilic acetotrophic methanogen *Methanothrix thermophila* CALS-1. *Arch Microbiol* **166**: 275-281.

Allen MA, Lauro FM, Williams TJ, Burg D, Siddiqui KS, De Francisci D, Chong KWY, Pilak O, Chew HH, De Maere MZ*, et al.* (2009) The genome sequence of the psychrophilic archaeon *Methanococcoides burtonii*: the role of genome evolution in cold adaptation. *ISME J* **3**: 1012-1035.

Andrews SC, Berks BC, McClay J, Ambler A, Quail MA, Golby P, Guest JR (1997) A 12-cistron *Escherichia coli* operon (*hyf*) encoding a putative proton-translocating formate hydrogenlyase system. *Microbiology* **143**: 3633-3647.

Bartoschek S, Vorholt JA, Thauer RK, Geierstanger BH, Griesinger C (2000) N-carboxymethanofuran (carbamate) formation from methanofuran and CO_2 in methanogenic archaea. Thermodynamics and kinetics of the spontaneous reaction. *Eur J Biochem* **267**: 3130-3138.

Battchikova N, Aro EM (2007) Cyanobacterial NDH-1 complexes: multiplicity in function and subunit composition. *Physiol Plant* **131**: 22-32.

Battchikova N, Eisenhut M, Aro EM (2011) Cyanobacterial NDH-1 complexes: Novel insights and remaining puzzles. *Biochim Biophys Acta* **1807**:935-944.

Battchikova N, Zhang P, Rudd S, Ogawa T, Aro EM (2005) Identification of NdhL and Ssl1690 (NdhO) in NDH-1L and NDH-1M complexes of *Synechocystis* sp. PCC 6803. *J Biol Chem* **280**: 2587-2595.

Bäumer S, Ide T, Jacobi C, Johann A, Gottschalk G, Deppenmeier U (2000) The $F_{420}H_2$ dehydrogenase from *Methanosarcina mazei* is a redox-driven proton pump closely related to NADH dehydrogenases. *J Biol Chem* **275**: 17968-17973.

Bäumer S, Murakami E, Brodersen J, Gottschalk G, Ragsdale SW, Deppenmeier U (1998) The $F_{420}H_2$:heterodisulfide oxidoreductase system from *Methanosarcina* species. 2-Hydroxyphenazine mediates electron transfer from $F_{420}H_2$ dehydrogenase to heterodisulfide reductase. *FEBS Lett* **428**: 295-298.

Becher B, Müller V, Gottschalk G (1992) N_5-methyl-tetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina strain* Gö1 is an Na^+-translocating membrane protein. *J Bacteriol* **174**: 7656-7660.

Beifuss U, Tietze M (2000) On the total synthesis of (S)-methanophenazine and the formal synthesis of (R)-methanophenazine from a common precursor. *Tetrahedron Lett* **41**: 9759-9763.

Beifuss U, Tietze M (2005) Methanophenazine and other natural biologically active phenazines. *Top Curr Chem* **244**: 77-113.

Berk H, Thauer RK (1998) $F_{420}H_2$: NADP oxidoreductase from *Methanobacterium thermoautotrophicum*: identification of the encoding gene via functional overexpression in *Escherichia coli*. *FEBS Lett* **438**: 124-126.

Biegel E, Müller V (2010) Bacterial Na^+-translocating ferredoxin:NAD^+ oxidoreductase. *Proc Natl Acad Sci U S A* **107**: 18138-18142.

Blaut M, Gottschalk G (1984) Coupling of ATP synthesis and methane formation from methanol and molecular hydrogen in *Methanosarcina barkeri*. *Eur J Biochem* **141**: 217-222.

Blaut M, Müller V, Gottschalk G (1987) Proton translocation coupled to methanogenesis from methanol + hydrogen in *Methanosarcina barkeri*. *FEBS Lett* **215**: 53-57.

Böhm R, Sauter M, Böck A (1990) Nucleotide sequence and expression of an operon in *Escherichia coli* coding for formate hydrogenlyase components. *Mol Microbiol* **4**: 231-243.

Bomsel JL, Pradet A (1968) Study of adenosine 5'-mono-, di- and triphosphates in plant tissues. IV. Regulation of the level of nucleotides, *in vivo*, by adenylate kinase: Theoretical and experimental study. *Biochim Biophys Acta* **162**: 230-242.

Boone DR, Whitman WB, Rouvière PE (1993). Diversity and taxonomy of methanogens. *Methanogenesis*. J. G. Ferry. New York, Chapman & Hall**:** 35-80.

Bott M, Thauer RK (1989) Proton translocation coupled to the oxidation of carbon monoxide to CO_2 and H_2 in *Methanosarcina barkeri*. *Eur J Biochem* **179**: 469-472.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Brodersen J, Bäumer S, Abken HJ, Gottschalk G, Deppenmeier U (1999a) Inhibition of membrane-bound electron transport of the methanogenic archaeon *Methanosarcina mazei* Gö1 by diphenyleneiodonium. *Eur J Biochem* **259**: 218-224.

Brodersen J, Gottschalk G, Deppenmeier U (1999b) Membrane-bound $F_{420}H_2$-dependent heterodisulfide reduction in *Methanococcus voltae*. *Arch Microbiol* **171**: 115-121.

Brüggemann H, Falinski F, Deppenmeier U (2000) Structure of the $F_{420}H_2$: quinone oxidoreductase of *Archaeoglobus fulgidus* - Identification and overproduction of the $F_{420}H_2$-oxidizing subunit. *Eur J Biochem* **267**: 5810-5814.

Clements AP, Kilpatrick L, Lu WP, Ragsdale SW, Ferry JG (1994) Characterization of the iron-sulfur clusters in ferredoxin from acetate-grown *Methanosarcina thermophila*. *J Bacteriol* **176**: 2689-2693.

de Poorter LM, Geerts WG, Theuvenet AP, Keltjens JT (2003) Bioenergetics of the formyl-methanofuran dehydrogenase and heterodisulfide reductase reactions in *Methanothermobacter thermautotrophicus*. *Eur J Biochem* **270**: 66-75.

Deckert G, Warren PV, Gaasterland T, Young WG, Lenox AL, Graham DE, Overbeek R, Snead MA, Keller M, Aujay M*, et al.* (1998) The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* **392**: 353-358.

Deppenmeier U (2002a) Redox-driven proton translocation in methanogenic archaea. *Cell Mol Life Sci* **59**: 1513-1533.

Deppenmeier U (2002b) The unique biochemistry of methanogenesis. *Prog Nucleic Acid Res Mol Biol* **71**: 223-283.

Deppenmeier U (2004) The membrane-bound electron transport system of *Methanosarcina* species. *J Bioenerg Biomembr* **36**: 55-64.

Deppenmeier U, Blaut M, Jussofie A, Gottschalk G (1988) A methyl-CoM methylreductase system from methanogenic bacterium strain Gö 1 not requiring ATP for activity. *FEBS Lett* **241**: 60-64.

Deppenmeier U, Blaut M, Mahlmann A, Gottschalk G (1990) Reduced coenzyme F_{420}: heterodisulfide oxidoreductase, a proton- translocating redox system in methanogenic bacteria. *Proc Natl Acad Sci U S A* **87**: 9449-9453.

Deppenmeier U, Blaut M, Schmidt B, Gottschalk G (1992) Purification and properties of a F_{420}-nonreactive, membrane-bound hydrogenase from *Methanosarcina* strain Gö1. *Arch Microbiol* **157**: 505-511.

Deppenmeier U, Johann A, Hartsch T, Merkl R, Schmitz RA, Martinez-Arias R, Henne A, Wiezer A, Bäumer S, Jacobi C*, et al.* (2002) The genome of *Methanosarcina mazei*: evidence for lateral gene transfer between bacteria and archaea. *J Mol Microbiol Biotechnol* **4**: 453-461.

Deppenmeier U, Müller V (2008) Life close to the thermodynamic limit: how methanogenic archaea conserve energy. *Results Probl Cell Differ* **45**: 123-152.

Deppenmeier U, Müller V, Gottschalk G (1996) Pathways of energy conservation in methanogenic archaea. *Arch Microbiol* **165**: 149-163.

Ding YH, Ferry JG (2004) Flavin mononucleotide-binding flavoprotein family in the domain *Archaea*. *J Bacteriol* **186**: 90-97.

Ebner S, Jaun B, Goenrich M, Thauer RK, Harmer J (2010) Binding of coenzyme B induces a major conformational change in the active site of methyl-coenzyme M reductase. *J Am Chem Soc* **132**: 567-575.

Efremov RG, Baradaran R, Sazanov LA (2010) The architecture of respiratory complex I. *Nature* **465**: 441-445.

Ehlers C, Weidenbach K, Veit K, Deppenmeier U, Metcalf WW, Schmitz RA (2005) Development of genetic methods and construction of a chromosomal *glnK1* mutant in *Methanosarcina mazei* strain Gö1. *Mol Genet Genomics* **273**: 290-298.

Eirich LD, Vogels GD, Wolfe RS (1979) Distribution of coenzyme F_{420} and properties of its hydrolytic fragments. *J Bacteriol* **140**: 20-27.

Elias DA, Juck DF, Berry KA, Sparling R (2000) Purification of the $NADP^+$: F_{420} oxidoreductase of *Methanosphaera stadtmanae*. *Can J Microbiol* **46**: 998-1003.

Ellermann J, Hedderich R, Böcher R, Thauer RK (1988) The final step in methane formation. Investigations with highly purified methyl-CoM reductase (component C) from *Methanobacterium thermoautotrophicum* (strain Marburg). *Eur J Biochem* **172**: 669-677.

Ellermann J, Kobelt A, Pfaltz A, Thauer RK (1987) On the role of N-7-mercaptoheptanoyl-O-phospho-L-threonine (component B) in the enzymatic reduction of methyl-coenzyme M to methane. *FEBS Lett* **220**: 358-362.

Ellman GL (1958) A colorimetric method for determining low concentrations of mercaptans. *Arch Biochem Biophys* **74**: 443-450.

Euro L, Belevich G, Verkhovsky MI, Wikström M, Verkhovskaya M (2008) Conserved lysine residues of the membrane subunit NuoM are involved in energy conversion by the proton-pumping NADH:ubiquinone oxidoreductase (Complex I). *Biochim Biophys Acta* **1777**: 1166-1172.

Fearnley IM, Walker JE (1992) Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins. *Biochim Biophys Acta* **1140**: 105-134.

Ferguson DJ Jr., Gorlatova N, Grahame DA, Krzycki JA (2000) Reconstitution of dimethylamine:coenzyme M methyl transfer with a discrete corrinoid protein and two methyltransferases purified from *Methanosarcina barkeri*. *J Biol Chem* **275**: 29053-29060.

Ferry JG (1997) Enzymology of the fermentation of acetate to methane by *Methanosarcina thermophila*. *Biofactors* **6**: 25-35.

Ferry JG (1999) Enzymology of one-carbon metabolism in methanogenic pathways. *FEMS Microbiol Rev* **23**: 13-38.

Ferry JG, Lessner DJ (2008) Methanogenesis in marine sediments. *Ann N Y Acad Sci* **1125**: 147-157.

Finel M (1998) Does NADH play a central role in energy metabolism in *Helicobacter pylori*? *Trends Biochem Sci* **23**: 412-413.

Fischer R, Thauer RK (1989) Methyltetrahydromethanopterin as an intermediate in methanogenesis from acetate in *Methanosarcina barkeri*. *Arch Microbiol* **151**: 459-465.

Fischer R, Thauer RK (1990) Ferredoxin-dependent methane formation from acetate in cell extracts of *Methanosarcina barkeri* (strain MS). *FEBS Lett* **269**: 368-372.

Fox JA, Livingston DJ, Orme-Johnson WH, Walsh CT (1987) 8-hydroxy-5-deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum*. 1. Purification and characterization. *Biochemistry* **26**: 4219-4227.

Fox JD, Kerby RL, Roberts GP, Ludden PW (1996) Characterization of the CO-induced, CO-tolerant hydrogenase from *Rhodospirillum rubrum* and the gene encoding the large subunit of the enzyme. *J Bacteriol* **178**: 1515-1524.

Franke-Whittle IH, Goberna M, Pfister V, Insam H (2009) Design and development of the ANAEROCHIP microarray for investigation of methanogenic communities. *J Microbiol Meth* **79**: 279-288.

Franzmann PD, Springer N, Ludwig W, Demacario EC, Rohde M (1992) A methanogenic archaeon from Ace Lake, Antarctica - *Methanococcoides burtonii* sp. nov. *Syst Appl Microbiol* **15**: 573-581.

Friedrich T, Scheide D (2000) The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. *FEBS Lett* **479**: 1-5.

Friedrich T, Steinmüller K, Weiss H (1995) The proton-pumping respiratory complex I of bacteria and mitochondria and its homolog in chloroplasts. *FEBS Lett* **367**: 107-111.

Friedrich T, Weiss H (1997) Modular evolution of the respiratory NADH:ubiquinone oxidoreductase and the origin of its modules. *J Theor Biol* **187**: 529-540.

Funk E, Schäfer E, Steinmüller K (1999) Characterization of the complex I-homologous NAD(P)H-plastoquinone-oxidoreductase (NDH-complex) of maize chloroplasts. *J Plant Physiol* **154**: 16-23.

Galagan JE, Nusbaum C, Roy A, Endrizzi MG, Macdonald P, FitzHugh W, Calvo S, Engels R, Smirnov S, Atnoor D*, et al.* (2002) The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. *Genome Res* **12**: 532-542.

Gencic S, Duin EC, Grahame DA (2010) Tight coupling of partial reactions in the acetyl-CoA decarbonylase/synthase (ACDS) multienzyme complex from *Methanosarcina thermophila*: acetyl C-C bond fragmentation at the A cluster promoted by protein conformational changes. *J Biol Chem* **285**: 15450-15463.

George DG, Hunt LT, Yeh LS, Barker WC (1985) New perspectives on bacterial ferredoxin evolution. *J Mol Evol* **22**: 20-31.

George GN, Prince RC, Mukund S, Adams MWW (1992) Aldehyde ferredoxin oxidoreductase from the hyperthermophilic archaebacterium *Pyrococcus furiosus* contains a tungsten oxo-thiolate center. *J Am Chem Soc* **114**: 3521-3523.

Gong W, Hao B, Wei Z, Ferguson DJ, Tallant T, Krzycki JA, Chan MK (2008) Structure of the alpha(2)epsilon(2) Ni-dependent CO dehydrogenase component of the *Methanosarcina barkeri* acetyl-CoA decarbonylase/synthase complex. *Proc Natl Acad Sci U S A* **105**: 9558-9563.

Gorris LGM, Voet ACWA, van der Drift C (1991) Structural characteristics of methanogenic cofactors in the non-methanogenic archaebacterium *Archaeoglobus fulgidus*. *Biofactors* **3**: 29-35.

Gottschalk G, Thauer RK (2001) The Na^+-translocating methyltransferase complex from methanogenic archaea. *Biochim Biophys Acta* **1505**: 28-36.

Guedeney G, Corneille S, Cuiné S, Peltier G (1996) Evidence for an association of *ndhB*, *ndhJ* gene products and ferredoxin-NADP-reductase as components of a chloroplastic NAD(P)H dehydrogenase complex. *FEBS Lett* **378**: 277-280.

Guss AM, Kulkarni G, Metcalf WW (2009) Differences in hydrogenase gene expression between *Methanosarcina acetivorans* and *Methanosarcina barkeri*. *J Bacteriol* **191**: 2826-2833.

Guss AM, Mukhopadhyay B, Zhang JK, Metcalf WW (2005) Genetic analysis of *mch* mutants in two *Methanosarcina* species demonstrates multiple roles for the methanopterin-dependent C_1 oxidation/reduction pathway and differences in H_2 metabolism between closely related species. *Mol Microbiol* **55**: 1671-1680.

Hagedoorn PL, Chen T, Schröder I, Piersma SR, de Vries S, Hagen WR (2005) Purification and characterization of the tungsten enzyme aldehyde:ferredoxin oxidoreductase from the hyperthermophilic denitrifier *Pyrobaculum aerophilum*. *J Biol Inorg Chem* **10**: 259-269.

Hall DO, Cammack R, Rao KK (1971) Role for ferredoxins in the origin of life and biological evolution. *Nature* **233**: 136-138.

Hartzell P, Reed DW (2006). The genus *Archaeoglobus*. *The Procaryotes*. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt. New York, Springer. **3:** 82-100.

Hedderich R, Forzi L (2005) Energy-converting [NiFe] hydrogenases: more than just H_2 activation. *J Mol Microbiol Biotechnol* **10**: 92-104.

Hedderich R, Hamann N, Bennati M (2005) Heterodisulfide reductase from methanogenic archaea: a new catalytic role for an iron-sulfur cluster. *Biol Chem* **386**: 961-970.

Heiden S, Hedderich R, Setzke E, Thauer RK (1994) Purification of a 2-subunit cytochrome-*b* containing heterodisulfide reductase from methanol-grown *Methanosarcina barkeri*. *Eur J Biochem* **221**: 855-861.

Hippe H, Caspari D, Fiebig K, Gottschalk G (1979) Utilization of trimethylamine and other N-methyl compounds for growth and methane formation by *Methanosarcina barkeri*. *Proc Natl Acad Sci U S A* **76**: 494-498.

Hoffman PS, Goodman TG (1982) Respiratory physiology and energy conservation efficiency of *Campylobacter jejuni*. *J Bacteriol* **150**: 319-326.

Hovey R, Lentes S, Ehrenreich A, Salmon K, Saba K, Gottschalk G, Gunsalus RP, Deppenmeier U (2005) DNA microarray analysis of *Methanosarcina mazei* Gö1 reveals adaptation to different methanogenic substrates. *Mol Genet Genomics* **273**: 225-239.

Hughes NJ, Chalk PA, Clayton CL, Kelly DJ (1995) Identification of carboxylation enzymes and characterization of a novel four-subunit pyruvate:flavodoxin oxidoreductase from *Helicobacter pylori*. *J Bacteriol* **177**: 3953-3959.

Hughes NJ, Clayton CL, Chalk PA, Kelly DJ (1998) *Helicobacter pylori porCDAB* and *oorDABC* genes encode distinct pyruvate:flavodoxin and 2-oxoglutarate:acceptor oxidoreductases which mediate electron transport to NADP. *J Bacteriol* **180**: 1119-1128.

Ide T, Bäumer S, Deppenmeier U (1999) Energy conservation by the H_2:heterodisulfide oxidoreductase from *Methanosarcina mazei* Gö1: identification of two proton-translocating segments. *J Bacteriol* **181**: 4076-4080.

Imlay JA (2006) Iron-sulphur clusters and the problem with oxygen. *Mol Microbiol* **59**: 1073-1082.

Jacobson FS, Daniels L, Fox JA, Walsh CT, Orme-Johnson WH (1982) Purification and properties of an 8-hydroxy-5-deazaflavin reducing hydrogenase from *Methanobacterium thermoautotrophicum*. *J Biol Chem* **257**: 3385-3388.

Jain N, Pathak H, Mitra S, Bhatia A (2004) Emission of methane from rice fields - A review. *J Sci Ind Res* **63**: 101-115.

Jeoung JH, Dobbek H (2007) Carbon dioxide activation at the Ni,Fe-cluster of anaerobic carbon monoxide dehydrogenase. *Science* **318**: 1461-1464.

Jetten MS, Fluit TJ, Stams AJ, Zehnder AJ (1992a) A fluoride-insensitive inorganic pyrophosphatase isolated from *Methanothrix soehngenii*. *Arch Microbiol* **157**: 284-289.

Jetten MS, Stams AJ, Zehnder AJ (1989) Isolation and characterization of acetyl-coenzyme A synthetase from *Methanothrix soehngenii*. *J Bacteriol* **171**: 5430-5435.

Jetten MSM, Stams AJM, Zehnder AJB (1992b) Methanogenesis from acetate - a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. *FEMS Microbiol Rev* **88**: 181-197.

Jussofie A, Mayer F, Gottschalk G (1986) Methane formation from methanol and molecular hydrogen by protoplasts of new methanogenic isolates and inhibition by dicyclohexylcarbodiimide. *Arch Microbiol* **146**: 245-249.

Kaesler B, Schönheit P (1989) The sodium cycle in methanogenesis. CO_2 reduction to the formaldehyde level in methanogenic bacteria is driven by a primary electrochemical potential of Na^+ generated by formaldehyde reduction to CH_4. *Eur J Biochem* **186**: 309-316.

Kamagata Y, Kawasaki H, Oyaizu H, Nakamura K, Mikami E, Endo G, Koga Y, Yamasato K (1992) Characterization of 3 thermophilic strains of *Methanothrix (Methanosaeta) thermophila* sp. nov. and rejection of *Methanothrix (Methanosaeta) thermoacetophila*. *Int J Syst Bacteriol* **42**: 463-468.

Kaneko T, Tabata S (1997) Complete genome structure of the unicellular cyanobacterium *Synechocystis* sp. PCC6803. *Plant Cell Physiol* **38**: 1171-1176.

Karakashev D, Batstone DJ, Angelidaki I (2005) Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Appl Environ Microbiol* **71**: 331-338.

Kashyap DR, Dadhich KS, Sharma SK (2003) Biomethanation under psychrophilic conditions: a review. *Bioresour Technol* **87**: 147-153.

Kaster AK, Moll J, Parey K, Thauer RK (2011) Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic methanogenic archaea. *Proc Natl Acad Sci U S A* **108**: 2981-2986.

Kawarabayasi Y, Hino Y, Horikawa H, Jin-no K, Takahashi M, Sekine M, Baba S, Ankai A, Kosugi H, Hosoyama A*, et al.* (2001) Complete genome sequence of an aerobic thermoacidophilic crenarchaeon, *Sulfolobus tokodaii* strain 7. *DNA Res* **8**: 123-140.

Kendall M, Boone D (2006). The order *Methanosarcinales*. *The Procaryotes*. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt. New York, Springer. **3:** 244-256.

Kerscher L, Oesterhelt D (1981) Purification and properties of two 2-oxoacid:ferredoxin oxidoreductases from *Halobacterium halobium*. *Eur J Biochem* **116**: 587-594.

Khalil MAK, Rasmussen RA (1994) Global emissions of methane during the last several centuries. *Chemosphere* **29**: 833-842.

Kimmich GA, Randles J, Brand JS (1975) Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. *Anal Biochem* **69**: 187-206.

Klenk HP, Clayton RA, Tomb JF, White O, Nelson KE, Ketchum KA, Dodson RJ, Gwinn M, Hickey EK, Peterson JD*, et al.* (1997) The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature* **390**: 364-370.

Kobelt A, Pfaltz A, Ankelfuchs D, Thauer RK (1987) The L-form of N-7-mercaptoheptanoyl-O-phosphothreonine is the enantiomer active as component B in methyl-CoM reduction to methane. *FEBS Lett* **214**: 265-268.

Krätzer C, Carini P, Hovey R, Deppenmeier U (2009) Transcriptional profiling of methyltransferase genes during growth of *Methanosarcina mazei* on trimethylamine. *J Bacteriol* **191**: 5108-5115.

Kulkarni G, Kridelbaugh DM, Guss AM, Metcalf WW (2009) Hydrogen is a preferred intermediate in the energy-conserving electron transport chain of *Methanosarcina barkeri*. *Proc Natl Acad Sci U S A* **106**: 15915-15920.

Künkel A, Vaupel M, Heim S, Thauer RK, Hedderich R (1997) Heterodisulfide reductase from methanol-grown cells of *Methanosarcina barkeri* is not a flavoenzyme. *Eur J Biochem* **244**: 226-234.

Kunow J, Linder D, Stetter KO, Thauer RK (1994) $F_{420}H_2$:quinone oxidoreductase from *Archaeoglobus fulgidus* - characterization of a membrane-bound multisubunit complex containing FAD and iron-sulfur clusters. *Eur J Biochem* **223**: 503-511.

Kunow J, Schwörer B, Stetter KO, Thauer RK (1993) A F_{420}-dependent NADP reductase in the extremely thermophilic sulfate-reducing *Archaeoglobus fulgidus*. *Arch Microbiol* **160**: 199-205.

Labes A, Schönheit P (2001) Sugar utilization in the hyperthermophilic, sulfate-reducing archaeon *Archaeoglobus fulgidus* strain 7324: starch degradation to acetate and CO_2 via a modified Embden-Meyerhof pathway and acetyl-CoA synthetase (ADP-forming). *Arch Microbiol* **176**: 329-338.

Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* **227**: 680-685.

Lee C, Kim J, Hwang K, O'Flaherty V, Hwang S (2009a) Quantitative analysis of methanogenic community dynamics in three anaerobic batch digesters treating different wastewaters. *Water Res* **43**: 157-165.

Lee M, Hidaka T, Hagiwara W, Tsuno H (2009b) Comparative performance and microbial diversity of hyperthermophilic and thermophilic co-digestion of kitchen garbage and excess sludge. *Bioresour Technol* **100**: 578-585.

Lessner DJ, Ferry JG (2007) The archaeon *Methanosarcina acetivorans* contains a protein disulfide reductase with an iron-sulfur cluster. *J Bacteriol* **189**: 7475-7484.

Lewalter K, Müller V (2006) Bioenergetics of archaea: Ancient energy conserving mechanisms developed in the early history of life. *Biochim Biophys Acta* **1757**: 437-445.

Li L, Li Q, Rohlin L, Kim U, Salmon K, Rejtar T, Gunsalus RP, Karger BL, Ferry JG (2007) Quantitative proteomic and microarray analysis of the archaeon *Methanosarcina acetivorans* grown with acetate versus methanol. *J Proteome Res* **6**: 759-771.

Lienard T, Becher B, Marschall M, Bowien S, Gottschalk G (1996) Sodium ion translocation by N_5-methyltetrahydromethanopterin: coenzyme M methyltransferase from *Methanosarcina mazei* Gö1 reconstituted in ether lipid liposomes. *Eur J Biochem* **239**: 857-864.

Lin XL, White RH (1986) Occurrence of coenzyme F_{420} and its gamma-monoglutamyl derivative in nonmethanogenic archaebacteria. *J Bacteriol* **168**: 444-448.

Livingston DJ, Fox JA, Orme-Johnson WH, Walsh CT (1987) 8-hydroxy-5-deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum*. 2. Kinetic and hydrogen-transfer studies. *Biochemistry* **26**: 4228-4237.

Lovley DR, Ferry JG (1985) Production and consumption of H_2 during growth of *Methanosarcina* spp. on acetate. *Appl Environ Microbiol* **49**: 247-249.

Ma BG, Chen L, Ji HF, Chen ZH, Yang FR, Wang L, Qu G, Jiang YY, Ji C, Zhang HY (2008a) Characters of very ancient proteins. *Biochem Biophys Res Commun* **366**: 607-611.

Ma KS, Thauer RK (1990) N_5,N_{10}-methylenetetrahydromethanopterin reductase from *Methanosarcina barkeri*. *FEMS Microbiol Lett* **70**: 119-124.

Ma W, Deng Y, Mi H (2008b) Redox of plastoquinone pool regulates the expression and activity of NADPH dehydrogenase supercomplex in *Synechocystis* sp. strain PCC 6803. *Curr Microbiol* **56**: 189-193.

Ma W, Deng Y, Ogawa T, Mi H (2006) Active NDH-1 complexes from the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Plant Cell Physiol* **47**: 1432-1436.

Madadi-Kahkesh S, Duin EC, Heim S, Albracht SP, Johnson MK, Hedderich R (2001) A paramagnetic species with unique EPR characteristics in the active site of heterodisulfide reductase from methanogenic archaea. *Eur J Biochem* **268**: 2566-2577.

Maeder DL, Anderson I, Brettin TS, Bruce DC, Gilna P, Han CS, Lapidus A, Metcalf WW, Saunders E, Tapia R, Sowers KR (2006) The *Methanosarcina barkeri* genome: comparative analysis with *Methanosarcina acetivorans* and *Methanosarcina mazei* reveals extensive rearrangement within methanosarcinal genomes. *J Bacteriol* **188**: 7922-7931.

Mahlmann A, Deppenmeier U, Gottschalk G (1989) Methanofuran-B is required for CO_2 formation from formaldehyde by *Methanosarcina barkeri*. *FEMS Microbiol Lett* **61**: 115-120.

Mai X, Adams MW (1996) Characterization of a fourth type of 2-keto acid-oxidizing enzyme from a hyperthermophilic archaeon: 2-ketoglutarate ferredoxin oxidoreductase from *Thermococcus litoralis*. *J Bacteriol* **178**: 5890-5896.

Massanz C, Schmidt S, Friedrich B (1998) Subforms and in vitro reconstitution of the NAD-reducing hydrogenase of *Alcaligenes eutrophus*. *J Bacteriol* **180**: 1023-1029.

Mathiesen C, Hägerhäll C (2002) Transmembrane topology of the NuoL, M and N subunits of NADH:quinone oxidoreductase and their homologues among membrane-bound hydrogenases and *bona fide* antiporters. *Biochim Biophys Acta* **1556**: 121-132.

Mayer F, Jussofie A, Salzmann M, Lübben M, Rohde M, Gottschalk G (1987) Immunoelectron microscopic demonstration of ATPase on the cytoplasmic membrane of the methanogenic bacterium strain Gö1. *J Bacteriol* **169**: 2307-2309.

McInerney MJ, Sieber JR, Gunsalus RP (2009) Syntrophy in anaerobic global carbon cycles. *Curr Opin Biotechnol* **20**: 623-632.

Metcalf WW, Zhang JK, Apolinario E, Sowers KR, Wolfe RS (1997) A genetic system for archaea of the genus *Methanosarcina*: Liposome-mediated transformation and construction of shuttle vectors. *Proc Natl Acad Sci U S A* **94**: 2626-2631.

Meuer J, Bartoschek S, Koch J, Künkel A, Hedderich R (1999) Purification and catalytic properties of Ech hydrogenase from *Methanosarcina barkeri*. *Eur J Biochem* **265**: 325-335.

Meuer J, Kuettner HC, Zhang JK, Hedderich R, Metcalf WW (2002) Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. *Proc Natl Acad Sci USA* **99**: 5632-5637.

Mi HL, Endo T, Ogawa T, Asada K (1995) Thylakoid membrane-bound, NADPH-specific pyridine-nucleotide dehydrogenase complex mediates cyclic electron-transport in the cyanobacterium *Synechocystis* sp. PCC-68038. *Plant Cell Physiol* **36**: 661-668.

Michel R, Massanz C, Kostka S, Richter M, Fiebig K (1995) Biochemical characterization of the 8-hydroxy-5-deazaflavin-reactive hydrogenase from *Methanosarcina barkeri* Fusaro. *Eur J Biochem* **233**: 727-735.

Mortenson LE (1964) Purification and analysis of ferredoxin from *Clostridium pasteurianum*. *Biochim Biophys Acta* **81**: 71-77.

Mott JE, Grant RA, Ho YS, Platt T (1985) Maximizing gene expression from plasmid vectors containing the Lambda-Pl promoter - strategies for overproducing transcription termination factor Rho. *Proc Natl Acad Sci U S A* **82**: 88-92.

Mukund S, Adams MW (1993) Characterization of a novel tungsten-containing formaldehyde ferredoxin oxidoreductase from the hyperthermophilic archaeon, *Thermococcus litoralis*. A role for tungsten in peptide catabolism. *J Biol Chem* **268**: 13592-13600.

Mukund S, Adams MWW (1995) Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem* **270**: 8389-8392.

Murakami E, Deppenmeier U, Ragsdale SW (2001) Characterization of the intramolecular electron transfer pathway from 2-hydroxyphenazine to the heterodisulfide reductase from *Methanosarcina thermophila*. *J Biol Chem* **276**: 2432-2439.

Muth E, Mörschel E, Klein A (1987) Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from the archaebacterium *Methanococcus voltae*. *Eur J Biochem* **169**: 571-577.

Nakamaru-Ogiso E, Kao MC, Chen H, Sinha SC, Yagi T, Ohnishi T (2010) The membrane subunit NuoL(ND5) is involved in the indirect proton pumping mechanism of Escherichia coli complex I. *J Biol Chem* **285**: 39070-39078.

Nakamura M, Saeki K, Takahashi Y (1999) Hyperproduction of recombinant ferredoxins in *Escherichia coli* by coexpression of the ORF1-ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 gene cluster. *J Biochem* **126**: 10-18.

Ng WV, Kennedy SP, Mahairas GG, Berquist B, Pan M, Shukla HD, Lasky SR, Baliga NS, Thorsson V, Sbrogna J*, et al.* (2000) Genome sequence of *Halobacterium* species NRC-1. *Proc Natl Acad Sci U S A* **97**: 12176-12181.

Nishizawa Y, Yabuki T, Fukuda E, Wakagi T (2005) Gene expression and characterization of two 2-oxoacid:ferredoxin oxidoreductases from *Aeropyrum pernix* K1. *FEBS Lett* **579**: 2319-2322.

Noll KM, Barber TS (1988) Vitamin contents of archaebacteria. *J Bacteriol* **170**: 4315-4321.

Noll KM, Donnelly MI, Wolfe RS (1987) Synthesis of 7-mercaptoheptanoylthreonine phosphate and its activity in the methylcoenzyme-M methylreductase system. *J Biol Chem* **262**: 513-515.

Noll KM, Rinehart KL, Tanner RS, Wolfe RS (1986) Structure of component-B (7-mercaptoheptanoylthreonine phosphate) of the methylcoenzyme M methylreductase system of *Methanobacterium thermoautotrophicum*. *Proc Natl Acad Sci U S A* **83**: 4238-4242.

Nowaczyk MM, Wulfhorst H, Ryan CM, Souda P, Zhang H, Cramer WA, Whitelegge JP (2011) NdhP and NdhQ: two novel small subunits of the cyanobacterial NDH-1 complex. *Biochemistry* **50**: 1121-1124.

Ohnishi T, Nakamaru-Ogiso E, Ohnishi ST (2010) A new hypothesis on the simultaneous direct and indirect proton pump mechanisms in NADH:quinone oxidoreductase (complex I). *FEBS Lett* **584**: 4131-4137.

Ott R (1959) Untersuchungen über Chinone. 2. Mitt.: Azinbildung durch Umsetzung von *p*-Chinonen mit *o*-Diaminen. *Mh. Chem.* **90**: 827-838.

Park MO, Mizutani T, Jones PR (2007) Glyceraldehyde-3-phosphate ferredoxin oxidoreductase from *Methanococcus maripaludis*. *J Bacteriol* **189**: 7281-7289.

Park YJ, Yoo CB, Choi SY, Lee HB (2006) Purifications and characterizations of a ferredoxin and its related 2-oxoacid:ferredoxin oxidoreductase from the hyperthermophilic archaeon, *Sulfolobus solfataricus* P1. *J Biochem Mol Biol* **39**: 46-54.

Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S*, et al.* (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**: 665-668.

Peck MW (1989) Changes in concentrations of coenzyme F_{420} analogs during batch growth of *Methanosarcina barkeri* and *Methanosarcina mazei*. *Appl Environ Microb* **55**: 940-945.

Peer CW, Painter MH, Rasche ME, Ferry JG (1994) Characterization of a CO:heterodisulfide oxidoreductase system from acetate-grown *Methanosarcina thermophila*. *J Bacteriol* **176**: 6974-6979.

Peinemann S, Blaut M, Gottschalk G (1989) ATP synthesis coupled to methane formation from methyl-CoM and H_2 catalyzed by vesicles of the methanogenic bacterial strain Gö1. *Eur J Biochem* **186**: 175-180.

Peinemann S, Müller V, Blaut M, Gottschalk G (1988) Bioenergetics of methanogenesis from acetate by *Methanosarcina barkeri*. *J Bacteriol* **170**: 1369-1372.

Peng LW, Shimizu H, Shikanai T (2008) The chloroplast NAD(P)H dehydrogenase complex interacts with photosystem I in *Arabidopsis*. *J Biol Chem* **283**: 34873-34879.

Pilkington SJ, Skehel JM, Gennis RB, Walker JE (1991) Relationship between mitochondrial NADH-ubiquinone reductase and a bacterial NAD-reducing hydrogenase. *Biochemistry* **30**: 2166-2175.

Pisa KY, Weidner C, Maischak H, Kavermann H, Müller V (2007) The coupling ion in the methanoarchaeal ATP synthases: H^+ vs. Na^+ in the A_1A_O ATP synthase from the archaeon *Methanosarcina mazei* Gö1. *FEMS Microbiol Lett* **277**: 56-63.

Pritchett MA, Metcalf WW (2005) Genetic, physiological and biochemical characterization of multiple methanol methyltransferase isozymes in *Methanosarcina acetivorans* C2A. *Mol Microbiol* **56**: 1183-1194.

Prommeenate P, Lennon AM, Markert C, Hippler M, Nixon PJ (2004) Subunit composition of NDH-1 complexes of *Synechocystis* sp. PCC 6803: identification of two new *ndh* gene products with nuclear-encoded homologues in the chloroplast Ndh complex. *J Biol Chem* **279**: 28165-28173.

Puchkaev AV, Ortiz de Montellano PR (2005) The *Sulfolobus solfataricus* electron donor partners of thermophilic CYP119: an unusual non-NAD(P)H-dependent cytochrome P450 system. *Arch Biochem Biophys* **434**: 169-177.

Ragsdale SW (2008) Catalysis of methyl group transfers involving tetrahydrofolate and B_{12}. *Vitam Horm* **79**: 293-324.

Ragsdale SW, Ljungdahl LG, der Vartanian DV (1983) Isolation of carbon monoxide dehydrogenase from *Acetobacterium woodii* and comparison of its properties with those of the *Clostridium thermoaceticum* enzyme. *J Bacteriol* **155**: 1224-1237.

Reay DS (2003) Sinking methane. *Biologist (London)* **50**: 15-19.

Reher M, Gebhard S, Schönheit P (2007) Glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) and nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN), key enzymes of the respective modified Embden-Meyerhof pathways in the hyperthermophilic crenarchaeota *Pyrobaculum aerophilum* and *Aeropyrum pernix*. *FEMS Microbiol Lett* **273**: 196-205.

Rohlin L, Gunsalus RP (2010) Carbon-dependent control of electron transfer and central carbon pathway genes for methane biosynthesis in the archaean *Methanosarcina acetivorans* strain C2A. *BMC Microbiol* **10**: 62.

Roy R, Adams MW (2002) Characterization of a fourth tungsten-containing enzyme from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Bacteriol* **184**: 6952-6956.

Roy R, Mukund S, Schut GJ, Dunn DM, Weiss R, Adams MW (1999) Purification and molecular characterization of the tungsten-containing formaldehyde ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*: the third of a putative five-member tungstoenzyme family. *J Bacteriol* **181**: 1171-1180.

Ruepp A, Graml W, Santos-Martinez ML, Koretle KK, Volker C, Mewes HW, Frishman D, Stocker S, Lupas AN, Baumeister W (2000) The genome sequence of the thermoacidophilic scavenger *Thermoplasma acidophilum*. *Nature* **407**: 508-513.

Rumeau D, Bécuwe-Linka N, Beyly A, Louwagie M, Garin J, Peltier G (2005) New subunits NDH-M, -N, and -O, encoded by nuclear genes, are essential for plastid Ndh complex functioning in higher plants. *Plant Cell* **17**: 219-232.

Sakuraba H, Ohshima T (2002) Novel energy metabolism in anaerobic hyperthermophilic archaea: A modified Embden-Meyerhof pathway. *J Biosci Bioeng* **93**: 441-448.

Sancho J (2006) Flavodoxins: sequence, folding, binding, function and beyond. *Cell Mol Life Sci* **63**: 855-864.

Sapra R, Bagramyan K, Adams MW (2003) A simple energy-conserving system: proton reduction coupled to proton translocation. *Proc Natl Acad Sci U S A* **100**: 7545-7550.

Sauter M, Böhm R, Böck A (1992) Mutational analysis of the operon (*hyc*) determining hydrogenase 3 formation in *Escherichia coli. Mol Microbiol* **6**: 1523-1532.

Schönheit P, Keweloh H, Thauer RK (1981) Factor F_{420} degradation in *Methanobacterium thermoautotrophicum* during exposure to oxygen. *FEMS Microbiol Lett* **12**: 347-349.

Selengut JD, Haft DH (2010) Unexpected abundance of coenzyme F_{420}-dependent enzymes in *Mycobacterium tuberculosis* and other actinobacteria. *J Bacteriol* **192**: 5788-5798.

Selig M, Xavier KB, Santos H, Schönheit P (1997) Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium *Thermotoga*. *Arch Microbiol* **167**: 217-232.

She Q, Singh RK, Confalonieri F, Zivanovic Y, Allard G, Awayez MJ, Chan-Weiher CCY, Clausen IG, Curtis BA, de Moors A*, et al.* (2001) The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. *Proc Natl Acad Sci U S A* **98**: 7835-7840.

Shibata M, Ohkawa H, Kaneko T, Fukuzawa H, Tabata S, Kaplan A, Ogawa T (2001) Distinct constitutive and low-CO_2-induced CO_2 uptake systems in cyanobacteria: genes involved and their phylogenetic relationship with homologous genes in other organisms. *Proc Natl Acad Sci U S A* **98**: 11789-11794.

Shin SG, Lee S, Lee C, Hwang K, Hwang S (2010) Qualitative and quantitative assessment of microbial community in batch anaerobic digestion of secondary sludge. *Bioresour Technol* **101**: 9461-9470.

Simianu M, Murakami E, Brewer JM, Ragsdale SW (1998) Purification and properties of the heme- and iron-sulfur-containing heterodisulfide reductase from *Methanosarcina thermophila*. *Biochemistry* **37**: 10027-10039.

Sirpiö S, Allahverdiyeva Y, Holmström M, Khrouchtchova A, Haldrup A, Battchikova N, Aro EM (2009) Novel nuclear-encoded subunits of the chloroplast NAD(P)H dehydrogenase complex. *J Biol Chem* **284**: 905-912.

Smith KS, Ingram-Smith C (2007) *Methanosaeta*, the forgotten methanogen? *Trends Microbiol* **15**: 150-155.

Smith MA, Finel M, Korolik V, Mendz GL (2000) Characteristics of the aerobic respiratory chains of the microaerophiles *Campylobacter jejuni* and *Helicobacter pylori*. *Arch Microbiol* **174**: 1-10.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76-85.

Soboh B, Linder D, Hedderich R (2002) Purification and catalytic properties of a CO-oxidizing:H_2-evolving enzyme complex from *Carboxydothermus hydrogenoformans*. *Eur J Biochem* **269**: 5712-5721.

Stojanowic A, Hedderich R (2004) CO_2 reduction to the level of formylmethanofuran in *Methanosarcina barkeri* is non-energy driven when CO is the electron donor. *FEMS Microbiol Lett* **235**: 163-167.

Supaphol S, Jenkins SN, Intomo P, Waite IS, O'Donnell AG (2011) Microbial community dynamics in mesophilic anaerobic co-digestion of mixed waste. *Bioresour Technol* **102**: 4021-4027.

Tagawa K, Arnon DI (1968) Oxidation-reduction potentials and stoichiometry of electron transfer in ferredoxins. *Biochim Biophys Acta* **153**: 602-613.

Tallant TC, Paul L, Krzycki JA (2001) The MtsA subunit of the methylthiol:coenzyme M methyltransferase of *Methanosarcina barkeri* catalyses both half-reactions of corrinoid-dependent dimethylsulfide: coenzyme M methyl transfer. *J Biol Chem* **276**: 4485-4493.

te Brömmelstroet BWJ, Geerts WJ, Keltjens JT, van der Drift C, Vogels GD (1991) Purification and properties of 5,10-methylenetetrahydromethanopterin dehydrogenase and 5,10-methylenetetrahydromethanopterin reductase, 2 coenzyme-F_{420}-dependent enzymes, from *Methanosarcina barkeri*. *Biochim Biophys Acta* **1079**: 293-302.

Terlesky KC, Ferry JG (1988) Ferredoxin requirement for electron transport from the carbon monoxide dehydrogenase complex to a membrane-bound hydrogenase in acetate-grown *Methanosarcina thermophila*. *J Biol Chem* **263**: 4075-4079.

Tersteegen A, Hedderich R (1999) *Methanobacterium thermoautotrophicum* encodes two multisubunit membrane-bound [NiFe] hydrogenases. Transcription of the operons and sequence analysis of the deduced proteins. *Eur J Biochem* **264**: 930-943.

Tersteegen A, Linder D, Thauer RK, Hedderich R (1997) Structures and functions of four anabolic 2-oxoacid oxidoreductases in *Methanobacterium thermoautotrophicum*. *Eur J Biochem* **244**: 862-868.

Thauer RK (1998) Biochemistry of methanogenesis: a tribute to Marjory Stephenson. *Microbiology* **144**: 2377-2406.

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* **41**: 100-180.

Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Microbiol* **6**: 579-591.

Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA*, et al.* (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**: 539-547.

Torres-Bacete J, Nakamaru-Ogiso E, Matsuno-Yagi A, Yagi T (2007) Characterization of the NuoM (ND4) subunit in *Escherichia coli* NDH-1: conserved charged residues essential for energy-coupled activities. *J Biol Chem* **282**: 36914-36922.

Torres-Bacete J, Sinha PK, Castro-Guerrero N, Matsuno-Yagi A, Yagi T (2009) Features of subunit NuoM (ND4) in *Escherichia coli* NDH-1: topology and implications of conserved Glu-144 for coupling site 1. *J Biol Chem* **284**: 33062-33069.

Tran-Betcke A, Warnecke U, Bocker C, Zaborosch C, Friedrich B (1990) Cloning and nucleotide sequences of the genes for the subunits of NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16. *J Bacteriol* **172**: 2920-2929.

van Beelen P, Dijkstra AC, Vogels GD (1983) Quantitation of coenzyme F_{420} in methanogenic sludge by the use of reversed phase high performance liquid chromatography and a fluorescence detector. *Eur J Appl Microbiol* **18**: 67-69.

van der Oost J, Schut G, Kengen SWM, Hagen WR, Thomm M, de Vos WM (1998) The ferredoxin-dependent conversion of glyceraldehyde-3-phosphate in the hyperthermophilic archaeon *Pyrococcus furiosus* represents a novel site of glycolytic regulation. *J Biol Chem* **273**: 28149-28154.

Vignais PM, Billoud B, Meyer J (2001) Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* **25**: 455-501.

Wächtershäuser G (1988) Before enzymes and templates: theory of surface metabolism. *Microbiol Rev* **52**: 452-484.

Walsh C (1986) Naturally occurring 5-deazaflavin coenzymes - biological redox roles. *Acc Chem Res* **19**: 216-221.

Weerakoon DR, Olson JW (2008) The *Campylobacter jejuni* NADH:ubiquinone oxidoreductase (complex I) utilizes flavodoxin rather than NADH. *J Bacteriol* **190**: 915-925.

Welte C, Deppenmeier U (2011) Re-evaluation of the function of the F_{420} dehydrogenase in electron transport in *Methanosarcina mazei*. *FEBS J* **278**: 1277-1287.

Welte C, Kallnik V, Grapp M, Bender G, Ragsdale S, Deppenmeier U (2010a) Function of Ech hydrogenase in ferredoxin-dependent, membrane-bound electron transport in *Methanosarcina mazei*. *J Bacteriol* **192**: 674-678.

Welte C, Krätzer C, Deppenmeier U (2010b) Involvement of Ech hydrogenase in energy conservation of *Methanosarcina mazei*. *FEBS J* **277**: 3396-3403.

Wilms R, Freiberg C, Wegerle E, Meier I, Mayer F, Müller V (1996) Subunit structure and organization of the genes of the A_1A_O ATPase from the achaeon *Methanosarcina mazei* Gö1. *J Biol Chem* **271**: 18843-18852.

Wolfe RS (1985) Unusual coenzymes of methanogenesis. *Trends Biochem Sci* **10**: 396-399.

Yamazaki S, Tsai L (1980) Purification and properties of 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from *Methanococcus vannielii*. *J Biol Chem* **255**: 6462-6465.

Zahler WL, Cleland WW (1968) A specific and sensitive assay for disulfides. *J Biol Chem* **243**: 716-719.

Zhang P, Battchikova N, Jansen T, Appel J, Ogawa T, Aro EM (2004) Expression and functional roles of the two distinct NDH-1 complexes and the carbon acquisition complex NdhD3/NdhF3/CupA/Sll1735 in *Synechocystis* sp PCC 6803. *Plant Cell* **16**: 3326-3340.

Zinder SH, Mah RA (1979) Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. *Appl Environ Microb* **38**: 996-1008.

Publications

Welte C, Hafner S, Krätzer C, Quentmeier A, Friedrich CG, Dahl C (2009) Interaction between Sox proteins of two physiologically distinct bacteria and a new protein involved in thiosulfate oxidation. *FEBS Lett* **583**:1281-1286.

Welte C, Kallnik V, Grapp M, Bender G, Ragsdale S, Deppenmeier U (2010) Function of Ech hydrogenase in ferredoxin-dependent, membrane-bound electron transport in *Methanosarcina mazei*. *J Bacteriol* **192**:674-678.

Welte C, Krätzer C, Deppenmeier U (2010) Involvement of Ech hydrogenase in energy conservation of *Methanosarcina maze*i. *FEBS J* **277**:3396-3403.

Krätzer C, Welte C, Dörner K, Friedrich T, Deppenmeier U (2011) Methanoferrodoxin represents a new class of superoxide reductase containing an iron-sulfur cluster. *FEBS J* **278**:442-451.

Welte C, Deppenmeier U (2011) Re-evaluation of the function of the F_{420} dehydrogenase in electron transport of *Methanosarcina mazei*. *FEBS J* **278**:1277-1287.

Welte C, Deppenmeier U (2011) Proton translocation in methanogens. *Methods Enzymol* **494**:257-280.

Welte C, Deppenmeier U (2011) Membrane-bound electron transport in *Methanosaeta thermophila*. *J Bacteriol* **193:**2868-2870.